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(57) Abstract

The present invention provides Human Papilloma Virus (HPV) fusion proteins, linked to an immunological fusion partner that provides T helper epiptopes to the HPV antigen. Vaccine formulations are provided that are useful in the treatment or Prophylaxis of HPV induced tumours.

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VACCINE

The present invention relates to vaccine compositions, comprising an E6 or/ and E7 or E6, E7 fusion protein from an HPV strain optionally linked with an immunological fusion partner and formulated with a CpG containing oligonucleotide into vaccines that find utility in the treatment or prophylaxis of human papilloma virus induced tumours or lesions. In particular, the present invention relates to vaccines comprising fusions proteins, comprising a protein or part of a protein that provides T helper epitopes (such as protein D from Haemophilus influenzae B) and an antigen from a human-papilloma virus (eg comprising an E6 or E7 protein from HPV 16 or 18 strain associated with cancer) that find utility in the treatment or prophylaxis of human papilloma induced tumours, wherein the vaccine is formulated with a CpG containing oligonucleotide as an adjuvant.

Papillomaviruses are small naked DNA tumour viruses (7.9 kilobases, double strand), which are highly species-specific. Over 70 individual human papillomavirus (HPV) genotypes have been described. Papillomaviruses are classified on the basis of species of origin (human, bovine etc.) and of the degree of genetic relatedness with other papillomaviruses from the same species. HPVs are generally specific for the skin or mucosal surfaces and have been broadly classified into "low" and "high" risk viruses.

Low risk HPVs usually cause benign ***lesions*** (warts or papillomas) that persist for several months or years. High risk HPVs are associated with pre-neoplastic lesions and cancer. The strongest positive association between an HPV virus and human cancer is that which exist between HPV 16 and 18 and cervical carcinoma. More than ten other HPV types have also been found in cervical carcinomas including HPV 31 and HPV 33 although at less frequency.

Genital HPV infection in young sexually active women is common and most individuals either clear the infection, or if lesions develop, these regress. Only a subset of infected individuals has lesions which progress to high grade intraephithelial neoplasia and only a fraction of these progress further to invasive carcinoma.

The molecular events leading to HPV infection have not been clearly established. The lack of an adequate *in vitro* system to propagate human

papillomaviruses has hampered the progress to a best information about the viral cycle.

Today, the different types of HPVs have been isolated and characterised with the help of cloning systems in bacteria and more recently by PCR amplification. The molecular organisation of the HPV genomes has been defined on a comparative basis with that of the well characterised bovine papillomavirus type 1 (BPV1).

Although minor variations do occur, all HPVs genomes described have at least seven early genes, E1 to E7 and two late genes L1 and L2. In addition, an upstream regulatory region harbors the regulatory sequences which appears to control most transcriptional events of the HPV genome.

E1 and E2 genes are involved in viral replication and transcriptional control, respectively and tend to be disrupted by viral integration. E6 and E7 are involved in viral transformation. E5 has also been implicated in this process.

In the HPVs involved in cervical carcinoma such as HPV 16 and 18, the oncogenic process starts after integration of viral DNA. The integration results in the inactivation of genes coding for the capsid proteins L1 and L2 and loss of E2 repressor function leads to deregulation of the E6/E7 open reading frame installing continuously overexpression of the two early proteins E6 and E7 that will lead to gradually loss of the normal cellular differentiation and the development of the carcinoma. E6 and E7 overcome normal cell cycle by inactivating major tumor suppressor proteins, p53 and pRB, the retinoblastoma gene product, respectively.

Carcinoma of the cervix is common in women and develops through a pre-cancerous intermediate stage to the invasive carcinoma which frequently leads to death. The intermediate stages of the disease is known as cervical intraepithelial neoplasia and is graded I to III in terms of increasing severity *(CIN I-III).*

Clinically, HPV infection of the female anogenital tract manifests as cervical flat condylomas, the hallmark of which is the koilocytosis affecting predominantly the superficial and intermediate cells of the cervical squamous epithelium.

Koilocytes which are the consequence of a cytopathic effect of the virus, appear as multinucleated cells with a perinuclear clear haloe. The epithelium is thickened with abnormal keratinisation responsible for the warty appearance of the lesion.

Such flat condylomas when positive for the HPV 16 or 18 serotypes, are high-risk factors for the evolution toward cervical intraepithelial neoplasia (CIN) and carcinoma in situ (CIS) which are themselves regarded as precursor lesions of invasive cervix carcinoma.

The natural history of oncogenic HPV infection presents three consecutive phases, namely:

(1) a latent infection phase,

(2) a phase of intranuclear viral replication with product of complete virions, which corresponds to the occurrence of koilocytes. At this stage, the HPV is producing its full range of proteins including E2, E5, E6, E7, L1 and L2.

(3) a phase of viral integration into the cellular genome, which triggers the onset of malignant transformation, and corresponds to CIN II and CIN III/CIS with progressive disappearance of koilocytes. At this stage, the expression of E2 is down-regulated, the expression of E6 and E7 is enhanced. Between CIN II/III and CIN III / Cervix carcinoma the viral DNA changes from being episomal in the basal cells to integration of E6 and E7 genes only (tumoral cells). 85% of all cervix carcinomas are squamous cell carcinomas most predominantly related to the HPV16 serotype. 10% and 5% are adenocarcinomas and adenosquamous cell carcinomas respectively, and both types are predominantly related to HPV 18 serotype. Nevertheless other oncogenic HPV's exist.

International Patent Application No. WO 96/19496 discloses variants of human papilloma virus E6 and E7 proteins, particularly fusion proteins of E6/E7 with a deletion in both the E6 and E7 proteins. These deletion fusion proteins are said to be immunogenic.

Immunomodulatory oligonucleotides contain unmethylated CpG dinucleotides ("CpG") and are known (WO 96/02555, EP 468520). CpG is an abbreviation for cytosine-guanosine dinucleotide motifs present in DNA. Historically, it was observed that the DNA fraction of BCG could exert an anti-tumour effect. In further studies, synthetic oligonucleotides derived from BCG gene sequences were shown to be capable of inducing immunostimulatory effects (both in vitro and in vivo). The authors of these studies concluded that certain palindromic sequences, including a central CG motif, carried this activity. The central role of the CG motif in

immunostimulation was later elucidated in a publication by Krieg, Nature 374, p546 1995. Detailed analysis has shown that the CG motif has to be in a certain sequence context, and that such sequences are common in bacterial DNA but are rare in vertebrate DNA.

It is currently believed that this evolutionary difference allows the vertebrate immune system to detect the presence of bacterial DNA (as occurring during an infection) leading consequently to the stimulation of the immune system. The immunostimulatory sequence as defined by Krieg is:

Purine Purine CG pyrimidine pyrimidine and where the CG motif is not methylated. In certain combinations of the six nucleotides a palindromic sequence is present. Several of these motifs, either as repeats of one motif or a combination of different motifs, can be present in the same oligonucleotide. The presence of one or more of these immunostimulatory sequence containing oligonucleotides can activate various immune subsets, including natural killer cells (which produce interferon γ and have cytolytic activity) and macrophages (Wooldrige et al Vol 89 (no. 8), 1977). Although other unmethylated CpG containing sequences not having this consensus sequence have now been shown to be immunomodulatory.

The present invention provides compositions comprising either an E6 or/and E7 or an E6/E7 fusion protein optionally linked to an immunological fusion partner having T cell epitopes, and adjuvanted with an immunomodulatory CpG containing oligonucleotide.

In a preferred form of the invention, the immunological fusion partner is derived from protein D of Heamophilus influenza B. Preferably the protein D derivative comprises approximately the first 1/3 of the protein, in particular approximately the first N-terminal 100-110 amino acids. The protein D may be lipidated (Lipo Protein D). Other immunological fusion partners include the non-structural protein from influenzae virus, NS1 (hemagglutinin). Typically the N terminal 81 amino acids are utilised, although different fragments may be used provided they include T-helper epitopes.

In another embodiment the immunological fusion partner is the protein known as LYTA. Preferably the C terminal portion of the molecule is used. Lyta is derived from Streptococcus pneumoniae which synthesize an N-acetyl-L-alanine amidase,

amidase LYTA, (coded by the lytA gen {Gene, 43 (1986) page 265-272} an autolysin that specifically degrades certain bonds in the peptidoglycan backbone. The C-terminal domain of the LYTA protein is responsible for the affinity to the choline or to some choline analogues such as DEAE. This property has been exploited for the development of E.coli C-LYTA expressing plasmids useful for expression of fusion proteins. Purification of hybrid proteins containing the C-LYTA fragment at its amino terminus has been described {Biotechnology: 10, (1992) page 795-798}. As used herein a preferred embodiment utilises the repeat portion of the Lyta molecule found in the C terminal end starting at residue 178. A particularly preferred form incorporates residues 188 - 305.

Accordingly, the present invention in preferred embodiment provides compositions comprising an immunomodulatory CpG oligonucleotide and a fusion proteins comprising Protein D - E6 from HPV 16, Protein D - E7 from HPV 16 Protein D - E7 from HPV 18, Protein D - E6 from HPV 18, and Protein D E6 E7 from both HPV 16 and 18. The protein D part preferably comprises the first 1/3 of protein D. It will be appreciated that other E6 and E7 proteins may be utilised from other HPV subtypes.

The proteins utilised in the present invention preferably are expressed in E. coli. In a preferred embodiment the proteins are expressed with a Histidine tail comprising between 5 to 9 and preferably six Histidine residues. These are advantageous in aiding purification.

The protein E7 may in a preferred embodiment carry one or several mutations in the binding site for the rb (retinoblastoma gene product) and hence eliminate any potential transforming capacity. Preferred mutations for HPV 16 E7 involve replacing Cys_{24} with Glycine, or Glutamic $acid_{26}$ with Glutamine. In a preferred embodiment the E7 protein contains both these mutations.

Preferred mutations for the HPV 18 E_7 involve replacing Cys_{27} with Glycine and/or Glutamic $acid_{29}$ with Glutamine. Again preferably both mutations are present.

Single or double mutations may also be introduced p53 region of E_6 to eliminate any potential transforming ability.

In a further embodiment of the invention there is provided and E6 E7 fusion protein from HPV linked to an immunological fusion partner and a CpG immunomodulatory oligonucleotide.

The vaccine of the present invention preferentially induce a TH1 immune response.

Two main types of Helper T cells have been characterized TH1 and TH2, which differ in the type of cytokines they secrete. These cytokines can be considered as the driving force behind the development of 2 different types of immune response : TH1-type of immune response is associated with cell mediated effector mechanisms such as production of the INF-γ and IL-2 cytokines by T-lymphocytes. INF-γ which in turn can activate other cells and induce them to secrete other important cytokines and mediators (INF-γ - activated NK cells produce IL12, IL2-activated NK cells are transformed into lymphokine activated killer cell (LAK), INF-γ-activated macrophages secrete inflamatory mediators like TNFa, IL1, IL6 and release nitric oxyde , IL2 can provide help for the differentiation of antigen specific, haplotype restricted cytotoxic T lymphocytes (CTL). At the antibody level , in mice, Th1-type of immune response is also associated with the generation of antibodies of the IgG2 isotype (IgG2a in Balb/c mice and IgG2b in C57BL/6 mice) .

The Th2-type of immune response is associated with a humoral immune response to the antigen. with the production of cytokines like IL4, IL5, IL6, IL10 and by the generation of a broad range of immunoglobulin isotypes including in mice IgG1, IgA, and IgM.

In man the distinction of Th1 and Th2-type immune responses is not absolute. An individual will support an immune response which is predominantly Th1 or predominantly Th2. However, it is often convenient to consider the families of cytokines in terms of that described in murine CD4 +ve T cell clones by Mosmann and Coffman (*Mosmann, T.R. and Coffman, R.L. (1989) TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annual Review of Immunology, 7, p145-173*).

In the human TH1 type of response is also associated with the presence of cytokine (IFNg and IL2) eventually with the presence of CTl and IgG2 isotypes in mice correspond to IgG1 type antibodies

This type 1 phenotype is of particular importance in protecting against viral and intracellular bacterial infections as well as in the treatment of cancer.

To manufacture the proteins used in the invention by recombinant techniques, an expression strategy can be used which involves fusion of E7, E6 or E6/E7 fusion to the 1/3-N-terminal portion of protein D from Haemophilus influenzae B, an immunological fusion partner providing T cell helper epitopes. An affinity polyhistidine tail is engineered at the carboxy terminus of the fusion protein allowing for simplified purification. Such recombinant antigen is overexpressed in E. coli as insoluble protein.

The proteins of the invention my be coexpressed with thioredoxin in trans (TIT). Coexpression of thioredoxin in trans versus in cis is preferred to keep antigen free of thioredoxin without the need for protease. Thioredoxin coexpression eases the solubilisation of the proteins of the invention. Thioredoxin coexpression has also a significant impact on protein purification yield, on purified-protein solubility and quality.

The replicable expression vectors may be prepared in accordance with the invention, by cleaving a vector compatible with the host cell to provide a linear DNA segment having an intact replicon, and combining said linear segment with one or more DNA molecules which, together with said linear segment encode the desired product, such as the DNA polymer encoding the protein of the invention, or derivative thereof, under ligating conditions.

Thus, the DNA polymer may be preformed or formed during the construction of the vector, as desired.

The choice of vector will be determined in part by the host cell, which may be prokaryotic or eukaryotic but preferably is E. coli. Suitable vectors include plasmids, bacteriophages, cosmids and recombinant viruses.

The preparation of the replicable expression vector may be carried out conventionally with appropriate enzymes for restriction, polymerisation and ligation of the DNA, by procedures described in, for example, Maniatis *et al.* cited above.

The recombinant host cell is prepared, in accordance with the invention, by transforming a host cell with a replicable expression vector of the invention under transforming conditions. Suitable transforming conditions are conventional and are

described in, for example, Maniatis *et al.* cited above, or "DNA Cloning" Vol. II, D.M. Glover ed., IRL Press Ltd, 1985.

The choice of transforming conditions is determined by the host cell. Thus, a bacterial host such as E. coli may be treated with a solution of $CaCl_2$ (Cohen *et al.*, Proc. Nat. Acad. Sci., 1973, *69*, 2110) or with a solution comprising a mixture of RbCl, $MnCl_2$, potassium acetate and glycerol, and then with 3-[N-morpholino]-propane-sulphonic acid, RbCl and glycerol. Mammalian cells in culture may be transformed by calcium co-precipitation of the vector DNA onto the cells. The invention also extends to a host cell transformed with a replicable expression vector of the invention.

Culturing the transformed host cell under conditions permitting expression of the DNA polymer is carried out conventionally, as described in, for example, Maniatis *et al.* and "DNA Cloning" cited above. Thus, preferably the cell is supplied with nutrient and cultured at a temperature below 50°C.

The product is recovered by conventional methods according to the host cell. Thus, where the host cell is bacterial, such as E. coli it may be lysed physically, chemically or enzymatically and the protein product isolated from the resulting lysate. Where the host cell is mammalian, the product may generally be isolated from the nutrient medium or from cell free extracts. Conventional protein isolation techniques include selective precipitation, adsorption chromatography, and affinity chromatography including a monoclonal antibody affinity column.

When the proteins of the present invention are expressed with a hisitidine tail (His tag). The proteins can easily be purified by affinity chromatography using an ion metal affinity chromatography column (IMAC) column.

A second chromatographic step, such as Q-sepharose may be utilised either before or after the IMAC column to yield highly purified protein. If the immunological fusion partner is C-LYTA, then it is possible to exploit the affinity of CLYTA for choline and/or DEAE to purify this product. Products containing both C-LYTA and his tags can be easily and efficiently purified in a two step process involving differential affinity chromatography. One step involves the affinity of the His tag to IMAC columns, the other involves the affinity of the C-terminal domain of LYTA for choline or DEAE.

A preferred vaccine composition comprises at least Protein D - E6 from HPV 16 or derivative thereof together with Protein D - E7 from HPV 16. Alternatively the E6 and E7 may be presented in a single molecule, preferably a Protein D E6/E7 fusion. Such vaccine may optionally contain either or both E6 and E7 proteins from HPV 18, preferably in the form of a Protein D - E6 or Protein D - E7 fusion protein or Protein D E6/E7 fusion protein. The vaccines of the present invention may contain other HPV antigens from HPV 16 or 18. In particular, the vaccine may contain L1 or L2 antigen monomers. Alternatively such L1 or L2 antigens may be presented together as a virus like particle or the L1 alone protein may be presented as virus like particle or caposmer structure. Such antigens, virus like particles and capsomer are per se known. See for example WO94/00152, WO94/20137, WO94/05792, and WO93/02184. Additional early proteins may be included such as E2 or preferably E5 for example The vaccine of the present invention may additionally comprise antigens from other HPV strains, preferably from strains HPV 6, 11, 31 or 33.

Vaccine preparation is generally described in Vaccine Design - The subunit and adjuvant approach (Ed. Powell and Newman) Pharmaceutical Biotechnology Vol. 6 Plenum Press 1995. Encapsulation within liposomes is described by Fullerton, US Patent 4,235,877.

The preferrred oligonucleotides preferably contain two or more CpG motifs separated by six or more nucleotides. The oligonucleotides of the present invention are typically deoxynucleotides. In a preferred embodiment the internucleotide in the oligonucleotide is phosphorodithioate, or more preferably a phosphorothioate bond, although phosphodiester and other internucleotide bonds are within the scope of the invention including oligonucleotides with mixed internucleotide linkages.

Preferred oligonucleotides have the following sequences: The sequences preferably contain all phosphorothioate modified internucleotide linkages.

OLIGO 1: TCC ATG ACG TTC CTG ACG TT

OLIGO 2: TCT CCC AGC GTG CGC CAT

OLIGO 3; ACC GAT GAC GTC GCC GGT GAC GGC ACC ACG

The CpG oligonucleotides utilised in the present invention may be synthesized by any method known in the art (eg EP 468520). Conveniently, such oligonucleotides may be synthesized utilising an automated synthesizer. Methods for producing

phosphorothioate oligonucleotides or phosphorodithioate are described in US5,666,153, US5,278,302 and WO95/26204.

The invention will be further described by reference to the following examples:

EXAMPLE I: Construction of an E. coli strain expressing fusion Protein-D1/3 - E7 -His (HPV16)

1) - Construction of expression plasmid

a) - Plasmid **pMG MCS prot D1/3** (= pRIT14589) is a derivative of pMG81 (described in UK patent application n° 951 3261.9 published as WO97/01640) in which the codons 4-81 of NS1 coding region from Influenza were replaced by the codons corresponding to residues Ser 20 → Thr127 of mature protein D of Haemophilus Influenzae strain 772, biotype 2 (H. Janson *et al.*, 1991. Infection and Immunity, Jan. p.119-125). The sequence of Prot-D1/3 is followed by a multiple cloning site (11 residues) and a coding region for a C-terminal histidine tail (6 His). This plasmid is used to express the fusion protein D1/3-E7-His.

b) - HPV genomic **E6 and E7 sequences** type **HPV 16** (See Dorf *et al.*, Virology 1985, 145, p. 181-185) were amplified from HPV 16 full length genome cloned in pBR322 (obtained from Deutsches Krebsforschungszentrum (DKFZ), Referenzzentrum für human pathogen Papillomaviruses - D 69120 - Heidelberg) and were subcloned into pUC19 to give TCA 301 (= pRIT14462).

Construction of plasmid TCA 308 (= pRIT14501): a plasmid expressing the fusion Protein-D1/3-E7-His

The nucleotides sequences corresponding to amino acids 1 → 98 of E7 protein are amplified from pRIT14462. During the polymerase chain reaction, NcoI and SpeI restriction sites were generated at the 5' and 3' ends of the E7 sequences allowing insertion into the same sites of plasmid pMGMCS Prot D1/3 to give plasmid TCA308 (= pRIT14501). The insert was sequenced to verify that no modification had been generated during the polymerase chain reaction. The sequence for the fusion protein-D1/3-E7-His (HPV 16) is described in sequence ID No.1 and the coding sequence in ID No.2.

2) - Transformation of AR58 strain

Plasmid pRIT14501 was introduced into E. coli AR58 (Mott *et al.*, 1985, Proc. Natl. Acad. Sci., 82:88) a defective λ lysogen containing a thermosensitive repressor of the λ pL promoter.

3) - Growth and induction of bacterial strain - Expression of Prot -D1/3-E7-His

Cells of AR58 transformed with plasmid pRIT14501 were grown in **100 ml** of LB medium supplemented with 50 µgr/ml of Kanamycin at 30°C. During the logarithmic phase of growth bacteria were shifted to 39°C to inactivate the λ repressor and turn on the synthesis of protein D1/3-E7-His. The incubation at 39°C was continued for 4 hours. Bacteria were pelleted and stored at -20°C.

EXAMPLE II: Construction of an *E.coli* strain expressing fusion Protein-D1/3-E6-his / HPV16

1. Construction of expression plasmid

a) Plasmid pMG MCS prot D1/3 (= pRIT14589) is a derivative of pMG81 (described in WO97/01640 in which the codons 4-81 of NS1 coding region from Influenza were replaced by the codons corresponding to residues Ser 20 → Thr 127 of mature protein D of Haemophilus Influenzae strain 772, biotype 2 (H. Janson *et al.*, 1991, Infection and Immunity, Jan. p.119-125). The sequence of Prot-D1/3 is followed by a multiple cloning site (11 residues) and a coding region for a C-terminal histidine tail (6 His). This plasmid is used to express the fusion protein D1/3-E6-his.

b) HPV genomic E6 and E7 sequences type HPV16 (Seedorf *et al.*, Virology 1985, 145, p.181-185) were amplified from HPV16 full length genome cloned in pBR322 (obtained from Deutsches Krebsforschungszentrum (DKFZ), Referenzzentrum für human pathogen Papillomaviruses -

c) D 69120 - Heidelberg) and were subcloned into pUC19 to give TCA 301 (= pRIT14462).

Construction of plasmid TCA 307 (=pRIT14497) : a plasmid expressing the fusion Protein-D1/3-E6-His /HPV16

The nucleotides sequences corresponding to amino acid.

1 → 151 of E6 protein were amplified from pRIT14462. During the polymerase chain reaction, NcoI and SpeI restriction sites were generated at the 5' and 3' ends of the E6 sequences allowing insertion into the same sites of plasmid pMGMCS Prot D1/3 to give plasmid TCA307 (= pRIT14497). The insert was

sequenced to verify that no modification had been generated during the polymerase chain reaction. The protein and coding sequence for the fusion protein-D1/3-E6-His is described in sequence ID No.3 and 4.

2. Transformation of AR58 strain

Plasmid pRIT14497 was introduced into *E. coli* AR58 (Mott et al., 1985, Proc. Natl. Acad. Sci., 82:88) a defective λ lysogen containing a thermosensitive repressor of the λ pL promoter.

3. Growth and induction of bacterial strain - Expression of Prot-D1/3-E6-His

Cells of AR58 transformed with plasmid pRIT14497 were grown in 100 ml of LB medium supplemented with 50 µgr/ml of Kanamycin at 30°C. During the logarithmic phase of growth bacteria were shifted to 39°C to inactivate the λ repressor and turn on the synthesis of protein D1/3-E6-his. The incubation at 39°C was continued for 4 hours. Bacteria were pelleted and stored at -20C.

4. Characterization of fusion Protein D1/3-E6-his (HPV 16)

Preparation of extracts

Frozen cells are thawed and resuspended in 10 ml of PBS buffer. Cells are broken in a French pressure cell press SLM Aminco at 20.000 psi (three passages). The extract is centrifuged at 16.000 g for 30 minutes at 4°C.

Analysis on Coomassie-stained SDS-polyacrylamide gels and Western blots

After centrifugation of extracts described above, aliquots of supernatant and pellet were analysed by SDS-polyacrylamide gel electrophoresis and Western blotting.

A major band of about 32 kDa, localized in the pellet fraction, was visualised by Coomassie stained gels and identified in Western blots by rabbit polyclonal anti-protein-D and by Ni-NTA conjugate coupled to calf intestinal alkaline phosphatase (Qiagen cat. n° 34510) which detects accessible histidine tail. The level of expression represents about 5 % of total protein.

5. Coexpression with thioredoxin

In an analagons fashion to the expression of prot D 1/3 E7 His from HPV 18 (example IX) an *E.coli* strain AR58 was transformed with a plasmid encoding thioredoxin and protein D 1/3 E7 His (HPV 16).

EXAMPLE III: Construction of an *E. coli* strain expressing fusion Protein-D1/3-E6E7-his / HPV16

1. Construction of expression plasmid

a) Plasmid pMG MCS prot D1/3 (= pRIT14589) is a derivative of pMG81 (described Supra) in which the codons 4-81 of NS1 coding region from Influenza were replaced by the codons corresponding to residues Ser 20 → Thr 127 of mature protein D of Haemophilus Influenzae strain 772, biotype 2 (H. Janson *et al.*, 1991, Infection and Immunity, Jan. p.119-125). The sequence of Prot-D1/3 is followed by a multiple cloning site (11 residues) and a coding region for a C-terminal histidine tail (6 His). This plasmid is used to express the fusion protein D1/3-E6E7-his.

b) HPV genomic E6 and E7 sequences type HPV16 (Seedorf *et al.*, Virology 1985, 145, p.181-185) were amplified from HPV16 full length genome cloned in pBR322 (obtained from Deutsches Krebsforschungszentrum (DKFZ), Referenzzentrum für human pathogen Papillomaviruses - D 69120 - Heidelberg) and were subcloned into pUC19 to give TCA 301 (= pRIT14462).

c) The coding sequences for E6 and E7 in TCA301 (= pRIT 14462) were modified with a synthetic oligonucleotides adaptor (inserted between Afl III and Nsi I sites) introducing a deletion of 5 nucleotides between E6 and E7 genes to remove the stop codon of E6 and create fused E6 and E7 coding sequences in the plasmid TCA309(= pRIT 14556).

Construction of plasmid TCA 311(= pRIT14512) : a plasmid expressing the fusion Protein-D1/3-E6E7-His /HPV16

The nucleotides sequences corresponding to amino acids 1 → 249 of fused E6E7 protein were amplified from pRIT14556. During the polymerase chain reaction, NcoI and SpeI restriction sites were generated at the 5' and 3' ends of the E6E7 fused sequences allowing insertion into the same sites of plasmid pMGMCS Prot D1/3 to give plasmid TCA311 (= pRIT14512). The insert was sequenced to verify that no modification had been generated during the polymerase chain reaction. The protein and coding sequence for the fusion protein-D E6/E7 1/3-His is described sequence ID No. 5 and 6.

2. Transformation of AR58 strain

Plasmid pRIT14512 was introduced into *E. coli* AR58 (Mott et al., 1985, Proc. Natl. Acad. Sci., 82:88) a defective λ lysogen containing a thermosensitive repressor of the λ pL promoter.

3. Growth and induction of bacterial strain - Expression of Prot-D1/3-E6E7-His

Cells of AR58 transformed with plasmid pRIT14512 were grown in 100 ml of LB medium supplemented with 50 µgr/ml of Kanamycin at 30°C. During the logarithmic phase of growth bacteria were shifted to 39°C to inactivate the λ repressor and turn on the synthesis of protein D1/3-E6E7-his. The incubation at 39°C was continued for 4 hours. Bacteria were pelleted and stored at -20C.

4. Characterization of fusion Protein D1/3-E6E7-his

Frozen cells are thawed and resuspended in 10 ml of PBS buffer. Cells are broken in a French pressure cell press SLM Aminco at 20.000 psi (three passages). The extract is centrifuged at 16.000 g for 30 minutes at 4°C.

After centrifugation of extracts described above, aliquots of supernatant and pellet were analysed by SDS-polyacrylamide gel electrophoresis and Western blotting.

A major band of about 48 kDa, localized in the pellet fraction, was visualised by Coomassie stained gels and identified in Western blots by rabbit polyclonal anti-protein-D and by Ni-NTA conjugate coupled to calf intestinal alkaline phosphatase (Qiagen cat. n° 34510) which detects accessible histidine tail. The level of expression represents about 1 % of total protein.

EXAMPLE: IV

In an analagous fashion the fusion protein of Lipo D 1/3 and E6-E7 from HPV16 was expressed in *E.coli* in the presence of thioredoxin.

The N-terminal of the pre-protein (388 aa) contains MDP residues followed by 16 amino acids of signal peptide of lipoprotein D (from Haemophilus Influenzae) which is cleaved in vivo to give the mature protein (370 aa). Lipoprotein portion (aa 1 to 127) is followed by the proteins E6 and E7 in fusion. The C terminal of the protein is elongated by TSGHHHHHH.

EXAMPLE V: Construction of E.coli strain B1002 expressing fusion ProtD1/3-E7

Mutated (cys24->gly,glu26->gln) type HPV16

1)-Construction of expression plasmid

Starting material:

a) - Plasmid pRIT 14501 (= TCA 308) which codes for fusion ProtD1/3-E7 -His

b) - Plasmid LITMUS 28 (New England Biolabs cat n° 306-28) , a cloning vector pUC-derived

c) - Plasmid pMG MCS ProtD1/3 (pRIT 14589) , a derivative of pMG81 (described Supra) in which the codons 4-81 of NS1 coding region from Influenza were replaced by the codons corresponding to residues Ser 20 → Thr 127 of mature protein D of Haemophilus Influenzae strain 772, biotype 2 (H. Janson *et al.*, 1991, Infection and Immunity, Jan. p.119-125). The sequence of Prot-D1/3 is followed by a multiple cloning site (11 residues) and a coding region for a C-terminal histidine tail (6 His)

Construction of plasmid pRIT 14733(=TCA347): a plasmid expressing the fusion Protein-D1/3-E7 mutated (cys24->gly ,glu26->gln) with His tail

The NcoI - XbaI fragment from pRIT 14501 (=TCA 308), bearing the coding sequence of E7 gene from HPV16 , elongated with an His tail , was subcloned in an intermediate vector Litmus 28 useful for mutagenesis to give pRIT 14909 (=TCA337) Double mutations **cys24-->gly** (Edmonds and Vousden , J.Virology 63 : 2650 (1989) and **glu26-->gln** (Phelps et al , J.Virology 66: 2418-27 (1992) were chosen to impair the binding to the antioncogene product of Retinoblastome gene (pRB). The introduction of mutations in E7 gene was realized with the kit " Quick Change Site directed Mutagenesis (Stratagene cat n° 200518) to give plasmid pRIT 14681(=TCA343) .After verification of presence of mutations and integrity of the complete E7 gene by sequencing , the mutated E7 gene was introduced into vector pRIT 14589 (= pMG MCS ProtD1/3) to give plasmid pRIT 14733 (=TCA347) protein and coding sequence.

The sequence for the fusion protein-D1/3-E 7 mutated (cys24->gly, glu26->gln) -His is described in sequence ID No. 7 and 8.

2)-Construction of strain B1002 expressing ProtD1/3-E7mutated (cys 24-->gly , glu26-->gln)-His /HPV16

Plasmid pRIT 14733 was introduced into *E.coli* **AR58** (Mott et al. ,1985,

Proc. Natl. Acad. Sci. , 82:88) a defective λ lysogen containing a thermosensitive repressor of the λ pL promoter ,to give strain B1002 , by selection for transformants resistant to kanamycine

3)-Growth and induction of bacterial strain B1002 - Expression of ProtD1/3-E7 mutated (cys 24->gly , glu26->gln)-His /HPV16

Cells of AR58 transformed with plasmid pRIT 14733 (B1002 strain) were grown at 30°C in 100 ml of LB medium supplemented with 50 µgr /ml of Kanamycin. During the logarithmic phase of growth bacteria were shifted to 39°C to inactivate the λ repressor and turn on the synthesis of ProtD1/3-E7 mutated -His /HPV16 . The incubation at 39°C was continued for 4 hours . Bacteria were pelleted and stored at -20°C.

4)-Characterization of fusion ProtD1/3-E7 mut (cys24->gly, glu26->gln)- His type HPV16.

Frozen cells were thawed and resuspended in 10 ml of PBS buffer.Cells were broken in a French Pressure cell press SLM Aminco at 20 000 psi (three passages) . The extract was centrifuged at 16000 g for 30 minutes at 4°C.

After centrifugation of extracts described above, aliquots of supernatant and pellet were analysed by SDS-polyacrylamide gel electrophoresis and Western blotting.

A major band of about 33 kDa, localized in the pellet fraction, was visualised by Coomassie stained gels and identified in Western blots by rabbit polyclonal 22 J 70 anti-protein D, by monoclonal anti E7 /HPV16 from Zymed and by Ni-NTA conjugate coupled to calf intestinal alkaline phosphatase (Qiagen cat. n° 34510) which detects accessible histidine tail. The level of expression represents about 3 to5 % of total protein.

Cells of B1002 were separated from the culture broth by centrifugation. The concentrated cells of B1002 were stored at -65°C.

EXAMPLE VI: Construction of an *E. coli* strain expressing fusion clyta-E6-his (HPV 16)

1. Construction of expression plasmid

a) -Plasmid pRIT14497 (= TCA307), that codes for fusion ProtD1/3-E6-His /HPV16

b)-Plasmid pRIT14661 (= DVA2), an intermediate vector containing the coding sequence for the 117 C-terminal codons of LytA of Streptococcus Pneumoniae. Lyta is derived from Streptococcus pneumoniae which synthesize an N-acetyl-L-alanine amidase, amidase LYTA. (coded by the lytA gene {Gene, 43 (1986) pag 265-272} an autolysin that specifically degrades certain bonds in the peptidoglycan backbone . The C-terminal domain of the LYTA protein is responsible for the affinity to the choline or to some choline analogues such as DEAE.

1.b Construction of plasmid pRIT14634 (=TCA332): a plasmid expressing the fusion clyta-E6-His /HPV16

a)The first step was the purification of the large NcoI-AflII restriction fragment from plasmid pRIT14497 and the purification of the small AflII-AflIII restriction fragment from pRIT14661

b)The second step was linking of clyta sequences to the E7-His sequences (NcoI and AflIII are compatible restriction sites) that gave rise to the plasmid pRIT 14634 (=TCA332), coding for the fusion protein clyta-E6-His under the control of the pL promoter.

The protein and coding sequence for the fusion protein clyta-E6-His is described sequence ID No. 9 and 10.

Transformation of AR58 strain

Plasmid pRIT14634 was introduced into *E. coli* AR58 (Mott et al., 1985, Proc. Natl. Acad. Sci., 82:88) a defective λ lysogen containing a thermosensitive repressor of the λ pL promoter.

Growth and induction of bacterial strain - Expression of clyta-E6-His

Cells of AR58 transformed with plasmid pRIT14634 were grown in 100 ml of LB medium supplemented with 50 µgr/ml of Kanamycin at 30°C. During the logarithmic phase of growth bacteria were shifted to 39°C to inactivate the λ repressor and turn on the synthesis of protein clyta-E6-his. The incubation at 39°C was continued for 4 hours. Bacteria were pelleted and stored at -20°C.

4. Characterization of fusion clyta-E6-his

Frozen cells were thawed and resuspended in 10 ml of PBS buffer. Cells were broken in a French pressure cell press SLM Aminco at 20.000 psi (three passages). The extract was centrifuged at 16.000 g for 30 minutes at 4°C. After centrifugation of

extracts described above, aliquots of supernatant and pellet were analysed by SDS-polyacrylamide gel electrophoresis and Western blotting.

A major band of about 33 kDa, localized in the pellet fraction, was visualised by Coomassie stained gels and identified in Western blots by rabbit polyclonal anti-clyta antibodies and by Ni-NTA conjugate coupled to calf intestinal alkaline phosphatase (Qiagen cat. n° 34510) which detects accessible histidine tail. The level of expression represents about 3 % of total protein.

EXAMPLE VII: Construction of an *E. coli* strain expressing fusion clyta-E7-his (HPV 16)

1. Construction of expression plasmid

1.a Starting materials

a) -Plasmid pRIT14501 (= TCA308), that codes for fusion ProtD1/3-E7-His /HPV16

b)-Plasmid pRIT14661 (= DVA2), an intermediate vector containing the coding sequence for the 117 C-terminal codons of LytA of Streptococcus Pneumoniae.

1.b Construction of plasmid pRIT14626 (=TCA330): a plasmid expressing the fusion clyta-E7-His / HPV16

a) The first step was the purification of the large NcoI-AflII restriction fragment from plasmid pRIT14501 and the purification of the small AflII-AflIII restriction fragment from pRIT14661

b) The second step was linking of clyta sequences to the E7-His sequences (NcoI and AflIII are compatible restriction sites) that gave rise to the plasmid pRIT 14626 (=TCA330), coding for the fusion protein clyta-E7-His under the control of the pL promoter.

The protein and coding sequence for the fusion protein clyta-E7-His is decribed in sequence ID No. 11 and 12.

2. Transformation of AR58 strain

Plasmid pRIT14626 was introduced into *E. coli* AR58 (Mott et al., 1985, Proc. Natl. Acad. Sci., 82:88) a defective λ lysogen containing a thermosensitive repressor of the λ pL promoter.

3. Growth and induction of bacterial strain - Expression of clyta-E7-His

Cells of AR58 transformed with plasmid pRIT14626 were grown in 100 ml of LB medium supplemented with 50 µgr/ml of Kanamycin at 30°C. During the

logarithmic phase of growth bacteria were shifted to 39°C to inactivate the λ repressor and turn on the synthesis of protein clyta-E7-his. The incubation at 39°C was continued for 4 hours. Bacteria were pelleted and stored at -20°C.

4. Characterization of fusion clyta-E7-his

Frozen cells were thawed and resuspended in 10 ml of PBS buffer. Cells were broken in a French pressure cell press SLM Aminco at 20.000 psi (three passages). The extract was centrifuged at 16.000 g for 30 minutes at 4°C. After centrifugation of extracts described above. aliquots of supernatant and pellet were analysed by SDS-polyacrylamide gel electrophoresis and Western blotting.

A major band of about 35 kDa, localized in the pellet fraction, was visualised by Coomassie stained gels and identified in Western blots by rabbit polyclonal anti-clyta antibodies and by Ni-NTA conjugate coupled to calf intestinal alkaline phosphatase (Qiagen cat. n° 34510) which detects accessible histidine tail. The level of expression represents about 5 % of total protein.

EXAMPLE VIII: Construction of an *E. coli* strain expressing fusion clyta-E6E7-his (HPV 16)

1. Construction of expression plasmid

1.a Starting materials

a) -Plasmid pRIT14512 (= TCA311), that codes for fusion ProtD1/3-E6E7-His /HPV16

b)-Plasmid pRIT14661 (= DVA2), an intermediate vector containing the coding sequence for the 117 C-terminal codons of LytA of Streptococcus Pneumoniae.

1.b Construction of plasmid pRIT14629 (=TCA331): a plasmid expressing the fusion clyta-E6E7-His /HPV16

a)The first step was the purification of the large NcoI-AflII restriction fragment from plasmid pRIT14512 and the purification of the small AflII-AflIII restriction fragment from pRIT14661

b)The second step was linking of clyta sequences to the E7-His sequences (NcoI and AflIII are compatible restriction sites)that gave rise to the plasmid pRIT 14629 (=TCA331), coding for the fusion protein clyta-E6E7-His under the control of the pL promoter.

The protein and coding sequence for the fusion protein clyta-E6E7-His is sequenced ID No. 13 and 14.

2. Transformation of AR58 strain

Plasmid pRIT14629 was introduced into *E. coli* AR58 (Mott et al., 1985, Proc. Natl. Acad. Sci., 82:88) a defective λ lysogen containing a thermosensitive repressor of the λ pL promoter.

3. Growth and induction of bacterial strain - Expression of clyta-E6E7-His

Cells of AR58 transformed with plasmid pRIT14629 were grown in 100 ml of LB medium supplemented with 50 µgr/ml of Kanamycin at 30°C. During the logarithmic phase of growth bacteria were shifted to 39°C to inactivate the λ repressor and turn on the synthesis of protein clyta-E6E7-his. The incubation at 39°C was continued for 4 hours. Bacteria were pelleted and stored at -20°C.

4. Characterization of fusion clyta-E6E7-his

Frozen cells were thawed and resuspended in 10 ml of PBS buffer. Cells were broken in a French pressure cell press SLM Aminco at 20.000 psi (three passages). The extract was centrifuged at 16.000 g for 30 minutes at 4°C.

After centrifugation of extracts described above, aliquots of supernatant and pellet were analysed by SDS-polyacrylamide gel electrophoresis and Western blotting.

A major band of about 48 kDa, localized in the pellet fraction, was visualised by Coomassie stained gels and identified in Western blots by rabbit polyclonal anti-clyta antibodies and by Ni-NTA conjugate coupled to calf intestinal alkaline phosphatase (Qiagen cat. n° 34510) which detects accessible histidine tail. The level of expression represents about 1 % of total protein.

EXAMPLE IX: Prot D1/3 E7 his (HPV 18) (E.Coli B1011)

Protein D1/3 E7 his HPV expressed with Thioredoxin inTrans (E.Coli B1012)

1) - Construction of expression plasmids

1).a.Construction of plasmid TCA316(=pRIT 14532) a plasmid expressing the fusion Protein-D1/3-E7-His /HPV18

Starting materials

a) - Plasmid pMG MCS prot D1/3 (= pRIT14589) is a derivative of pMG81 (described in UK patent application n° 951 3261.9 published as WO97/01640 in

which the codons 4-81 of NS1 coding region from Influenza were replaced by the codons corresponding to residues Ser 20 → Thr 127 of mature protein D of Haemophilus Influenzae strain 772, biotype 2 (H. Janson *et al.*, 1991, Infection and Immunity, Jan. p.119-125). The sequence of Prot-D1/3 is followed by a multiple cloning site (11 residues) and a coding region for a C-terminal histidine tail (6 His). This plasmid is used to express the fusion protein D1/3-E7-his.

b) - HPV genomic E6 and E7 sequences of prototype HPV18(Cole et al.J.Mol.Biol.(1987)193,599-608) were amplified from HPV16 full length genome cloned in pBR322 (obtained from Deutsche Krebsforschungszentrum (DKFZ), Referenzzentrum für human pathogen Papillomaviruses - D 69120 - Heidelberg) and were subcloned into pUC19 to give TCA 302 (= pRIT14467).

Construction of plasmid TCA 316(= pRIT14532)

The nucleotides sequences corresponding to amino acids 1 → 105 of E7 protein were amplified from pRIT14467. During the polymerase chain reaction, NcoI and SpeI restriction sites were generated at the 5' and 3' ends of the E7 sequences allowing insertion into the same sites of plasmid pMGMCS Prot D1/3 to give plasmid TCA316 (= pRIT14532). The insert was sequenced and a modification versus E7/HPV18 prototype sequence was identified in E7 gene (nucleotide 128 G->A) generating a substitution of a glycine by a glutamic acid (aa 43 in E7 , position 156 in fusion protein). The protein and coding sequence for the fusion protein-D1/3-E7-His /HPV18 is set forth in sequence ID No. 15 and ID No. 16.

1).b. Construction of plasmid TCA313 (=pRIT14523): a plasmid expressing thioredoxin

Starting materials

a) - Plasmid pBBR1MCS4(Antoine R. and C.Locht,Mol.Microbiol. 1992,6,1785-1799 ; M.E.Kovach et al. Biotechniques 16, (5), 800-802)which is compatible with plasmids containing ColE1 or P15a origins of replication.

b) - Plasmid pMG42 (described in WO93/04175) containing the sequence of promoter pL of Lambda phage

c) - Plasmid pTRX (Invitrogen, kit Thiofusion K350-01) bearing the coding sequence for thioredoxin followed by AspA transcription terminator.

Construction of plasmid TCA313(=pRIT14523)

The fragment EcoRI-NdeI fragment from pMG42, bearing pL promoter and the NdeI-HindIII fragment from pTRX, bearing the coding sequence for thioredoxin followed by AspA terminator, were purified and ligated into the EcoRI and HindIII sites of plasmid vector pBBR1MCS4 to give plasmid TCA313(= pRIT14523).

The coding sequence for thioredoxin is described in ID No. 17.

2) - Transformation of AR58 strain

2).a. To obtain strain B1011 expressing ProtD1/3-E7-His/HPV18

Plasmid pRIT14532 was introduced into *E. coli* AR58 (Mott et al., 1985. Proc. Natl. Acad. Sci., 82:88) a defective λ lysogen containing a thermosensitive repressor of the λ pL promoter , by selection for transformants resistant to kanamycine.

2).b. Construction of strain B1012 expressing ProtD1/3-E7-His/HPV18 and thioredoxin

Plasmid pRIT14532 and pRIT14523 were introduced into *E. coli* AR58 (Mott et al., 1985, Proc. Natl. Acad. Sci., 82:88) a defective λ lysogen containing a thermosensitive repressor of the λ pL promoter ,by double selection for transformants resistant to kanamycin and ampicillin.

3) - Growth and induction of bacterial strains B1011 and B1012 - Expression of Prot-D1/3-E7-His/HPV18 without and with thioredoxin in trans

Cells of AR58 transformed with plasmids pRIT14532 (B1011 strain) and Cells of AR58 transformed with plasmids pRIT14532 and pRIT14523 (B1012 strain) were grown at 30°c in 100 ml of LB medium supplemented with 50 µgr/ml of Kanamycin for B1011 strain and supplemented 50 µgr/ml of Kanamycin and 100 µgr/ml of Ampicillin for B1012 strain . During the logarithmic phase of growth bacteria were shifted to 39°C to inactivate the λ repressor and turn on the synthesis of protein D1/3-E7-his/HPV18 and thioredoxin. The incubation at 39°C was continued for 4 hours.

Characterization of fusion Protein D1/3-E7-his /HPV18

Preparation of extracts

Frozen cells are thawed and resuspended in 10 ml of PBS buffer. Cells are broken in a French pressure cell press SLM Aminco at 20.000 psi (three passages). The extract is centrifuged at 16.000 g for 30 minutes at 4°C.

Analysis on Coomassie-stained SDS-polyacrylamide gels and Western blots

After centrifugation of extracts described above, aliquots of supernatant and pellet were analysed by SDS-polyacrylamide gel electrophoresis and Western blotting.

The fusion protD1/3-E7-His (about 31 kDa) was visualised by Coomassie stained gels in the pellet fraction for strain B1011 and partially localized (30%) in the supernatant fraction for strain B1012 and was identified in Western blots by rabbit polyclonal anti-protein-D and by Ni-NTA conjugate coupled to calf intestinal alkaline phosphatase (Qiagen cat. n° 34510) which detects accessible histidine tail. The level of expression represents about 1-3% of total protein as shown on a Coomassie-stained SDS-polyacrylamide gel.

For the extract of strain B1012 the thioredoxin (about 12 KDa) was visualised by coomassie stained gel in the supernatant and identified in western blots by monoclonal anti thioredoxin (Invitrogen R920-25)

EXAMPLE X: Construction of E.coli strain B1098 expressing fusion ProtD1/3-E7

Mutated (cys27->gly,glu29->gln) type HPV18

1)-Construction of expression plasmid

Starting material:

a) - Plasmid pRIT 14532 (= TCA 316) which codes for fusion ProtD1/3-E7 -His

b) - Plasmid LITMUS 28 (New England Biolabs cat n° 306-28) , a cloning vector pUC-derived

c) - Plasmid pMG MCS ProtD1/3 (pRIT 14589) , a derivative of pMG81 (described supra) in which the codons 4-81 of NS1 coding region from Influenza were replaced by the codons corresponding to residues Ser 20 → Thr 127 of mature protein D of Haemophilus Influenzae strain 772, biotype 2 (H. Janson *et al.*, 1991, Infection and Immunity, Jan. p.119-125). The sequence of Prot-D1/3 is followed by a multiple cloning site (11 residues) and a coding region for a C-terminal histidine tail (6 His)

Construction of plasmid pRIT 14831(=TCA355): a plasmid expressing the fusion Protein-D1/3-E7 mutated (cys27->gly ,glu29->gln) with His tail

The NcoI - XbaI fragment from pRIT 14532 (=TCA 316), bearing the coding sequence of E7 gene from HPV18 , elongated with an His tail , was subcloned in an intermediate vector Litmus 28 useful for mutagenesis to give pRIT 14910 (=TCA348)

By analogy with E7/HPV16 mutagenesis, double mutations **cys27-->gly** and **glu29-->gln** were chosen to impair the binding to the antioncogene product of Retinoblastome gene (pRB).

The introduction of mutations in E7 gene was realized with the kit " Quick Change Site directed Mutagenesis (Stratagene cat n° 200518) .As the sequencing of pRIT14532 had pointed out the presence of a glutamic acid in position 43 of E7 instead of a glycine in the prototype sequence of HPV18 , a second cycle of mutagenesis was realized to introduce a glycine in position 43 . We obtained plasmid pRIT 14829 (= TCA353). After verification of presence of mutations and integrity of the complete E7 gene by sequencing , the mutated E7 gene was introduced into vector pRIT 14589 (= pMG MCS ProtD1/3) to give plasmid pRIT 14831 (=TCA355).

The protein and coding sequence for the fusion protein-D1/3-E 7 mutated (cys27->gly, glu29->gln) -His is described in sequence ID No. 18 and 19.

2)Construction of strain B1098 expressing ProtD1/3-E7mutated (cys 27-->gly , glu29-->gln)-His /HPV18

Plasmid pRIT 14831 was introduced into *E.coli* **AR58** (Mott et al. ,1985, Proc. Natl. Acad. Sci. , 82:88) a defective λ lysogen containing a thermosensitive repressor of the λ pL promoter ,to give strain B1098 , by selection for transformants resistant to kanamycin.

3)-Growth and induction of bacterial strain B1098 - Expression of ProtD1/3-E7 mutated (cys 27->gly , glu29->gln)-His /HPV18

Cells of AR58 transformed with plasmid pRIT 14831 (B1098 strain) were grown at 30°C in 100 ml of LB medium supplemented with 50 µgr /ml of Kanamycin. During the logarithmic phase of growth bacteria were shifted to 39°C to inactivate the λ repressor and turn on the synthesis of ProtD1/3-E7 mutated -His /HPV18 . The incubation at 39°C was continued for 4 hours. Bacteria were pelleted and stored at -20°C.

4)-Characterization of fusion ProtD1/3-E7 mut (cys24->gly, glu26->gln)- His type HPV16

Frozen cells were thawed and resuspended in 10 ml of PBS buffer. Cells were broken in a French Pressure cell press SLM Aminco at 20 000 psi (three passages) . The extract was centrifuged at 16000 g for 30 minutes at 4°C.

Analysis on Coomassie stained SDS-polyacrylamide gels and Western blots

After centrifugation of extracts described above, aliquots of supernatant and pellet were analysed by SDS-polyacrylamide gel electrophoresis and Western blotting. A major band of about 31 kDa, localized in the pellet fraction, was visualised by Coomassie stained gels and identified in Western blots by rabbit polyclonal 22 J 70 anti-protein D and by monoclonal Penta-His (Qiagen cat. n° 34660) which detects accessible histidine tail. The level of expression represents about 3 to5 % of total protein.

EXAMPLE XI: Construction of an *E. coli* strain expressing fusion Protein-D1/3-E6-his / HPV18

1. Construction of expression plasmid

a) Plasmid pMG MCS prot D1/3 (= pRIT14589) is a derivative of pMG81 (described supra) in which the codons 4-81 of NS1 coding region from Influenza were replaced by the codons corresponding to residues Ser 20 → Thr 127 of mature protein D of Haemophilus Influenzae strain 772, biotype 2 (H. Janson *et al.*, 1991, Infection and Immunity, Jan. p.119-125). The sequence of Prot-D1/3 is followed by a multiple cloning site (11 residues) and a coding region for a C-terminal histidine tail (6 His). This plasmid is used to express the fusion protein D1/3-E6-his.

HPV genomic E6 and E7 sequences type HPV18 (Cole et al., J. Mol. Biol. 1987, 193 , p.599-608.) were amplified from HPV18 full length genome cloned in pBR322 (obtained from Deutsches Krebsforschungszentrum (DKFZ), Referenzzentrum für human pathogen Papillomaviruses - D 69120 - Heidelberg) and were subcloned into pUC19 to give TCA 302 (= pRIT14467).

Construction of plasmid TCA 314(= pRIT14526) : a plasmid expressing the fusion Protein-D1/3-E6-His /HPV18

The nucleotides sequences corresponding to amino acids 1 → 158 of E6 protein were amplified from pRIT14467. During the polymerase chain reaction, NcoI and SpeI restriction sites were generated at the 5' and 3' ends of the E6 sequences allowing insertion into the same sites of plasmid

pMGMCS Prot D1/3 to give plasmid TCA314 (= pRIT14526). The insert was sequenced to verify that no modification had been generated during the polymerase chain reaction. The protein and coding sequence for the fusion protein-D1/3-E6-His is described in sequence ID No. 20 and 21.

Transformation of AR58 strain

Plasmid pRIT14526 was introduced into *E. coli* AR58 (Mott et al., 1985, Proc. Natl. Acad. Sci., 82:88) a defective λ lysogen containing a thermosensitive repressor of the λ pL promoter.

3. Growth and induction of bacterial strain - Expression of Prot-D1/3-E6-His

Cells of AR58 transformed with plasmid pRIT14526 were grown in 100 ml of LB medium supplemented with 50 µgr/ml of Kanamycin at 30°C. During the logarithmic phase of growth bacteria were shifted to 39°C to inactivate the λ repressor and turn on the synthesis of protein D1/3-E6-his. The incubation at 39°C was continued for 4 hours. Bacteria were pelleted and stored at -20C.

4. Characterization of fusion Protein D1/3-E6-his

Frozen cells are thawed and resuspended in 10 ml of PBS buffer. Cells are broken in a French pressure cell press SLM Aminco at 20.000 psi (three passages). The extract is centrifuged at 16.000 g for 30 minutes at 4°C. After centrifugation of extracts described above, aliquots of supernatant and pellet were analysed by SDS-polyacrylamide gel electrophoresis and Western blotting.

A major band of about 32 kDa, localized in the pellet fraction, was visualised by Coomassie stained gels and identified in Western blots by rabbit polyclonal anti-protein-D and by Ni-NTA conjugate coupled to calf intestinal alkaline phosphatase (Qiagen cat. n° 34510) which detects accessible histidine tail. The level of expression represents about 3-5 % of total protein.

EXAMPLE XII: Construction of an *E. coli* strain expressing fusion Protein-D1/3-E6E7-his / HPV18

***1.* Construction of expression plasmid**

a) Plasmid pMG MCS prot D1/3 (= pRIT14589) is a derivative of pMG81 (described supra) in which the codons 4-81 of NS1 coding region from Influenza were replaced by the codons corresponding to residues Ser 20 → Thr 127 of mature protein D of Haemophilus Influenzae strain 772, biotype 2 (H. Janson *et al.*, 1991, Infection and

Immunity, Jan. p.119-125). The sequence of Prot-D1/3 is followed by a multiple cloning site (11 residues) and a coding region for a C-terminal histidine tail (6 His). This plasmid is used to express the fusion protein D1/3-E6E7-his.

b) HPV genomic E6 and E7 sequences type HPV18 (Cole et al.,J.Mol.Biol. 1987, 193, 599-608) were amplified from HPV18 full length genome cloned in pBR322 (obtained from Deutsches Krebsforschungszentrum (DKFZ), Referenzzentrum für human pathogen Papillomaviruses - D 69120 - Heidelberg) and were subcloned into pUC19 to give TCA 302 (= pRIT14467).

c) The coding sequences for E6 and E7 in TCA302 (= pRIT 14467) were modified with a synthetic oligonucleotides adaptor (inserted between Hga I and Nsi I sites) introducing a deletion of 11 nucleotides between E6 and E7 genes, removing the stop codon of E6 and creating fused E6 and E7 coding sequences in the plasmid TCA320(= pRIT 14618).

Construction of plasmid TCA 328(= pRIT14567) : a plasmid expressing the fusion Protein-D1/3-E6E7-His /HPV18

The nucleotides sequences corresponding to amino acids

1 → 263 of fused E6E7 protein were amplified from pRIT14618. During the polymerase chain reaction, NcoI and SpeI restriction sites were generated at the 5' and 3' ends of the E6E7 fused sequences allowing insertion into the same sites of plasmid pMGMCS Prot D1/3 to give plasmid TCA328 (= pRIT14567). The insert was sequenced to verify that no modification had been generated during the polymerase chain reaction. The protein and coding sequence for the fusion protein-D1/3-E6E7-His is described in sequence ID No. 22 and 23.

2. Transformation of AR58 strain

Plasmid pRIT14567 was introduced into *E. coli* AR58 (Mott et al., 1985, Proc. Natl. Acad. Sci., 82:88) a defective λ lysogen containing a thermosensitive repressor of the λ pL promoter.

3. Growth and induction of bacterial strain - Expression of Prot-D1/3-E6E7-His

Cells of AR58 transformed with plasmid pRIT14512 were grown in 100 ml of LB medium supplemented with 50 µgr/ml of Kanamycin at 30°C. During the logarithmic phase of growth bacteria were shifted to 39°C to inactivate the λ repressor

and turn on the synthesis of protein D1/3-E6E7-his. The incubation at 39°C was continued for 4 hours. Bacteria were pelleted and stored at -20C.

4.Characterization of fusion Protein D1/3-E6E7-his

Frozen cells are thawed and resuspended in 10 ml of PBS buffer. Cells are broken in a French pressure cell press SLM Aminco at 20.000 psi (three passages). The extract is centrifuged at 16.000 g for 30 minutes at 4°C.

After centrifugation of extracts described above, aliquots of supernatant and pellet were analysed by SDS-polyacrylamide gel electrophoresis and Western blotting.

A major band of about 48 kDa, localized in the pellet fraction, was visualised by Coomassie stained gels and identified in Western blots by rabbit polyclonal anti-protein-D and by Ni-NTA conjugate coupled to calf intestinal alkaline phosphatase (Qiagen cat. n° 34510) which detects accessible histidine tail. The level of expression represents about 1 % of total protein.

EXAMPLE XIII

The therapeutic potential of vaccine containing the PD1/3 E7 fusion protein and different CpG oligonucleotides were evaluated in the TC1 (E7 expressing tumour model.)

1. **Therapeutic experiments: protocol**

10e6 TC1 cells, E7 expressing tumour cells: were injected subcutaneously (200µl) in the flank of C57BL/6 immunocompetent mice. Mice were vaccinated 7 and 14 days after the tumour challenge, with 5µg ProtD 1/3 E7 HPV16 injected intra-footpad (100µl : 50µl / footpad) in the presence of different adjuvants:

2 and 4 weeks after the second immunisation, 5 mice/group were killed and spleens or popliteal lymph nodes were taken and analyzed for immune response.

1.2 Results

Groups of mice

1) PBS
2) ProtD1/3 E7 HPV16
3) ProtD1/3 E7 HPV16 + oligo 1: 1826 (WD 1001): TCC ATG ACG TTC CTG ACG TT
4) Oligo 1
5) ProtD1/3 E7 HPV16 + oligo 2/ 1758 (WD1002): TCT CCC AGC GTG CGC CAT

6) Oligo 2

Tumour Growth;

was monitored by measuring individual tumours twice a week.

Figure 1 : represents the mean tumour growth (in mm2)/group n=10 followed over 4 weeks.

- The injection of 10e6 TC1 cells injected subcutaneously give rise to a growing tumour in 100% of the animals.
- Vaccinating with ProtD1/3E7 or adjuvant alone: 100% of the animals develop a tumour.
- As shown in figure 1 and 2, in the groups of mice that received the antigen with a CpG oligonucleotide the mean tumour growth remained very low and very similar between groups, reflecting that the tumour growth either was slowed down or that several tumours were completely rejected.

The analysis of individual tumour growth 2 and 4 weeks after the latest vaccination showed that complete rejection in the groups were:

	Day 28 (n=10)	**day 42 (n=5)**
E7+oligo1 (1826)	**40%**	**40%**
Oligo1	0%	0%
E7+oligo2 (1758)	**70%**	**40%**
Oligo2	0%	0%

The mean tumour growth/group of mice vaccinated with PD1/3 E7+ the CpG oligos are quite similar and analysis of the individual tumour growth showed that the CpG oligos induce prolonged complete tumour rejection.

Conclusion

Both CpG (Oligo 2>oligo 1) induced complete tumour regression .

Lymphoproliferative response was analysed by *in vitro* restimulation of spleen and lymph nodes cells for 72 hrs with either PD1/3E7, the protein E7(Bollen) and PD (whole) PD1/3 (coated or not on latex µbeads) (10, 1, 0.1 µg/ml) 2 and 4 weeks post II.

- Positive controls (ConA stimulaltion) were positive.

- Surprisingly, no E7 specific and no PD specific proliferative response could be observed starting with spleen cells 2 or 4 weeks post II (probably due to a technical problem: data not shown).
- On the contrary, lymph node cells from mice that received ProtD1/3 E7 in CpG oligos 1 and 2 showed a very good E7 specific proliferative response although almost no PD (whole) specific response could be observed even at the hightest concentration of 100µg/ml no PD1/3 specific responses was observed even when coated on latex µbeads.

Similar data were obtained 4 weeks post II.

Serology

The anti E7 antibody response: IgG tot and isotypes (IgG1, IgG2a, IgG2b, IgGTot) were measured by ELISA using the E7 protein as coating antigen as described in the Materials and Methods. Figures 3 and 4 show the relative percentage of the different IgG isotypes in the total of IgGs, 2 and 4 weeks post II respectively.

- The Oligos affect only weakly (oligo 2) or not at all (Oligo 1) the weak antibody response observed when PD1/3E7 alone was injected.
- The predominant E7 specific antibody subclass was clearly IgG2b for all the formulation tested (80-90% of the total IgGs).

The same results were obtained 4 weeks post II

Isotypic profile of anti E7 responses (post II, pooled sera) exp. 97293

Groups	IgG1	IgG2a	IgG2b	IgGtot
1) PBS	0	0	0	0
2) ProtD1/3 E7 HPV16	1020	0	4130	4740
3) ProtD1/3 E7 HPV16 + oligo 1	170	400	3680	4910
4) Oligo 1	0	0	530	420
5) ProtD1/3 E7 HPV16 + oligo 2	0	590	7560	13690
6) Oligo 2	0	0	0	0

Groups	IgG1	IgG2a	IgG2b	IgGtot
1) PBS	0	0	0	0
2) ProtD1/3 E7 HPV1	240	0	1650	1400
3) ProtD1/3 E7 HPV16 + oligo 1	0	0	1280	1430
4) Oligo 1	0	0	0	0
5) ProtD1/3 E7 HPV16 + oligo 2	0	560	3600	5880
6) Oligo 2	0	0	0	0

CTL assay:

A CTL response could be detected when measured 2 weeks after the latest vaccination, when cells were re-stimulated in vitro with irradiated TC1 when TC1 or peptide E7 pulsed EL4, were used as target cells, when mice immunised with PD1/3 E7 + CpG oligo 2> 1 (25-40% specific lysis) and not with oligos alone.

- Lysis was seen on TC1 cells than on peptide E7 pulsed EL4 cells, but this is mostly observed in the groups of mice vaccinated with PD1/3E7 + CpG oligos (2>1). In this experiment other formulations did not induce a CTL.
- Using E7 pulsed EL4 cells, no lysis was observed when mice received the protein or the adjuvant alone.

1.3 Materials and Methods

Component	Brand	Batch number	Concentration (mg/ml)	Buffer
ProtD1/3-E7		957/015	0.677	PBS 7.4
oligo CpG 1826	EuroGentec	WD1001	5	H_20
oligo CpG	EuroGentec	WD1002	5	H_20

1.3.1 Formulation Process

All the formulations were prepared on the day of injection.

Oligo containing formulations

Formulations containing oligo alone without other adjuvant were prepared by addition of CpG to the diluted PrtD1/3-E7 in PBS pH 7.4.

The adjuvant controls without antigen were prepared by replacing the protein by PBS.

1.3.2 Mice and Cell lines

Mice C57Bl/6 (Iffa Credo) 6-8 weeks old mice were used in these experiments.

Cell lines: TC1 (obtained from the John Hopkin's University) , or EL4 cells were grown in RPMI 1640 (Bio Whittaker) containig 10% FCS and additives: 2mM L-Glutamine , 1% antibiotics (10000U/ml penicilin. 10000µg/ml streptomycin) 1% non essential amino acid 100x, 1% sodium pyruvate (Gibco), 5 10e-5 M 2-

mercaptoethanol. Before injection TC1 cells were trypsynized and washed in serum free medium.

1.3.3 Tumour growth:

All the animals were injected with tumor cells on day O and were randomized at day 7. Individual tumor growth was followed over time (the 2 main diameters (A, B) were measured using calipers twice a week, A x B represents the "tumor surface" and the average of the 5 values / groups is showed on a graphic over time: 6 weeks

1.3.4 CMI read out

In vitro lymphoproliferation

Lymphoproliferation was performed on individual spleens and on lymph node pools. 200000 spleen cells or popliteal lymph node cells were plated in triplicate, in 96 well microplate, in RPMI medium containing 1% normal mice serum and additives . After 72 hrs of in vitro re-stimulation with different amounts of PD1/3 E7 (1, 0.1, 0.01 µg/ml) or E7 (10-1-0.1 µg/ml) After 72hrs, 100 µl of culture supernatant were removed and replaced by fresh medium containing 1µCi 3H thymidine (Amersham 5Ci/mmol) . After 16 hrs, cells were harvested onto filter plates. Incorporated radioactivity was counted in a β counter. Results are expressed in CPM (mean of triplicate wells) or as stimulation indexes (mean CPM in cultures with antigen / mean CPM in cultures without antigen).

1.3.5 CTL assay

20 10e6 spleen cells were co-cultured with 2 10e6 irradiated (18000r) TC1 cells (E7 expressing tumor) for 7 days in the presenced or absence of ConA sup. (2%)

Target cells used to assess cytotoxicity were either Cr51 (DuPont NEN 37MBq/ml) loaded (1hr at 37°C) TC1 cells or E7 pulsed EL4 cells (for 1 hr at 37°C during the Cr 51 loading of the cells10µg/ml of E7-derived peptide (49-57) (QCB) compared to EL4 cells NK dependant lysis was assessed on K562 target cells 2000 target cells were added / well of 96 well plate (V botttom nunc 2-45128) with 100/1 being the highest Effector / target ratio. Controls for spontaneous or maximal Cr51 release were performed in sextuplet and were targets in medium or in triton 1.5%. All plates were gently centrifuged and incubated for 4 hrs at 37 in 7% CO2. 50 µl of the

supernatant was deposed on 96w Lumaplate (Packard) let dry O/N and counted in a Top Count counter. Data is expressed as percent specific lysis which is calculated from the c.p.m. by the formula (experimental release - spontaneous release) / (maximal release - spontaneous release) X 100.

Serology

Quantitation of anti E7 antibody was performed by Elisa using E7as coating antigen. Antigen and antibody solutions were used at 50 µl per well. Antigen was diluted at a final concentration of 3 µg/ml in carbonate buffer ph9.5 and was adsorbed overnight at 4°c to the wells of 96 wells microtiter plates (Maxisorb Immuno-plate, Nunc, Denmark). The plates were then incubated for 1hr at 37°c with PBS containing 1% bovine serum albumin and 0.1% Tween 20 (saturation buffer). Two-fold dilutions of sera (starting at 1/100 dilution) in the saturation buffer were added to the E7-coated plates and incubated for 1 hr 30 min at 37°c. The plates were washed 3 times with PBS 0.1% Tween 20 and biotin-conjugated anti-mouse IgG1, IgG2a or IgG2b or IgGtot (Amersham, UK) diluted 1/5000 in saturation buffer was added to each well and incubated for 1 hr 30 min at 37°c. After a washing step, streptavidin-biotinylated peroxydase complex (Amersham, UK) diluted 1/5000 in saturation buffer was added for an additional 30 min at 37°c. Plates were washed as above and incubated for 10 min with TMB(tetra-methyl-benzidine). The reaction was stopped with H2SO4 4N and read at 450 nm. Midpoint dilutions were calculated by SoftmaxPro (using a four parameters equation).

EXAMPLE XIV

In a second experiment, the vaccine of the invention were tested to assess the significance of the backbone:

Therapeutic experiment: protocol

- 10e6 TC1 cells , E7 expressing tumor cells : were injected subcutaneously (200µl) in the flank of immunocompetent C57BL/6 mice.

- 2 vaccinations, 7 and 14 days after the tumor challenge, with 5µg ProtD 1/3 E7 HPV16 injected intra- footpad (100 µl : 50µl / footpad) +/- CpG oligo; Oligo 1 (WD1001) as a phosphorothioate modified or the same Oligo (WD1006) but with phosphodiester linkage.

- 5 animals /group.

The **tumor growth** was monitored by measuring individual tumors twice a week and the mean tumor growth/ group of 5 animals is depicted in figure 5 and show the phosphorothioate modified oligonucleotides are effective in bringing about tumour regression.

Conclusions:

- All the animals that received 10e6 TC1 tumor cells develop a growing tumor.
- 100% of the animals vaccinated twice, 7 days apart, with the PD1/3 E7 HPV16 protein alone develop a tumor.
- 100% of the animals receiving the PD1/3 E7 protein + oligo WD1006 develop a tumor at the concentrations tested
- All the groups of animals that received the E7 protein + CpG 1001 at a concentration ranging from 10 to 200µg show tumor regression partial or complete(20-40%).

The first concentration at which this therapeutic effect on tumor regression is not fully obtained is E7+ 1µg CpG oligo 1001.

EXAMPLE XV

In a third series of experiments, the vaccines of the invention were evaluated in transgenic mice expressing E7 protein.

- The transgenic mouse strain has been generated by M. Parmentier and C. Ledent at the IRIBHN (ULB). (Ref: PNAS (USA) 1990, 87; 6176-6180).
- As transgenic mice live with the E7 HPV16 gene from birth, they are considered "tolerant" to this gene: E7 from HPV 16, in this situation is considered as a "self antigen".

- The expression of the transgene is driven by the thyroglobulin promoter. As Thyroglobulin is constitutively expressed only In the Thyroid, E7 is expressed in the thyroid.

- As a consequence of this expression, thyroid cells proliferate, mouse develop goiter and nodules which after 6 months to 1 year can evoluate in invasive cancer.

The results (figure 6) of the experiments show that therapeutic vaccination with CpG oligonucleotide and antigen as described herein, results in a reduction of tumour growth and can induce complete tumour regression.

Material & Methods

- 10e6 TC1 cells, E7 expressing tumor cells : were injected subcutaneously (200µl) in the flank of male or female C57BL/6 Transgenic

- mice were vaccinated 7 and 14 days after the tumor challenge, with 5µg ProtD 1/3 E7 HPV16 injected intra- footpad (100 µl : 50µl / footpad) in the 2 presence of CpG oligonucleotide TCT CCC AGC GTG CGC CAT and two control adjuvants:,

- 10 animals /group

2 and 4 weeks after the second immunization were killed and spleens or popliteal lymph.

Conclusion

The vaccines of the invention are effective in bringing about tumour regression in HPV induced tumours.

CLAIMS

1. A composition comprising an E6 or E7 protein or E6/E7 fusion protein from HPV optionally linked to an immunological fusion partner, and an immunomodulatory CpG oligonucleotide.

2. A composition as claimed in claim 1 wherein the fusion partner is selected from the group; protein D or a fragment thereof from Heamophilius influenzae B, lipoprotein D or fragment thereof from Heamophilius influenzae B, NS1 or fragment thereof from Influenzae Virus, and LYTA or fragment thereof from Streptococcus Pneumoniae.

3. A composition as claimed in claim 1 or 2 wherein the E6 or E7 proteins are derived from HPV16 or HPV18.

4. A composition as claimed in claim 1, 2 or 3 wherein the E7 protein is mutated.

5. A composition as claimed in claim 1, 2 or 3 wherein the E6 protein is mutated.

6. A composition as claimed in any of claims 1 to 5 additionally comprising a histidine tag of at least 4 histidine residues.

7. A composition as claimed herein comprising an additional HPV antigen.

8. A composition as claimed herein where the immumodulatory CpG oligonucleotide comprises a hexamer motif: purine purine cytosine guaine pyrimidine pyrimidine.

9. A composition as claimed herein wherein the immunomodulatory CpG oligonucleotide has two or more CpG motifs.

10. A composition as claimed herein wherein the CpG oligonucleotide contains a phosphorothioate inter-nucleotide linkage.

11. A composition as claimed herein wherein the CpG oligonucleotide is selected from the group:

OLIGO 1: TCC ATG ACG TTC CTG ACG TT

OLIGO 2: TCT CCC AGC GTG CGC CAT

OLIGO 3: ACC GAT GAC GTC GCC GGT GAC GGC ACC ACG

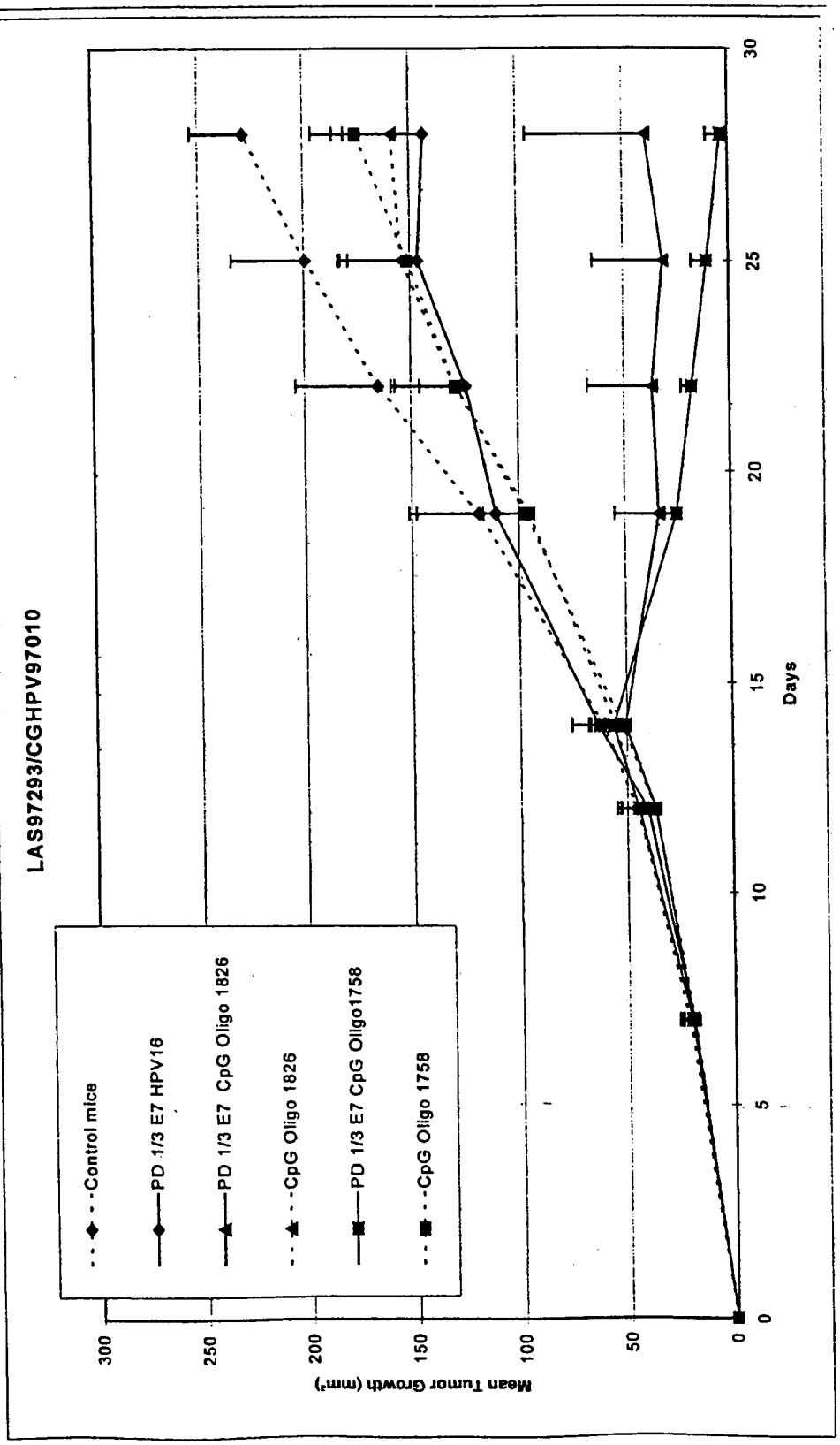
12. A composition as claimed herein for use in medicine.

13. A method of inducing an immune response in a patient to an HPV antigen comprising administering a safe and effective amount of a composition as claimed herein.

14. A method of preventing or treating HPV induced tumours in a patient comprising administering a safe and effective amount of a composition as claimed herein.

15. A method of preparing a composition as claimed herein, comprising admixing an E6, E7 or E6/E7 fusion protein optionally linked to an immunological fusion partner, and an immunomodulatory CpG oligonucleotide.

Fig. 1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Days
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50
100
150
200
250
300
0
5
10
15
20
25
30
- - ◆ - -Control mice
—◆— PD 1/3 E7 HPV16
—▲— PD 1/3 E7 CpG Oligo 1826
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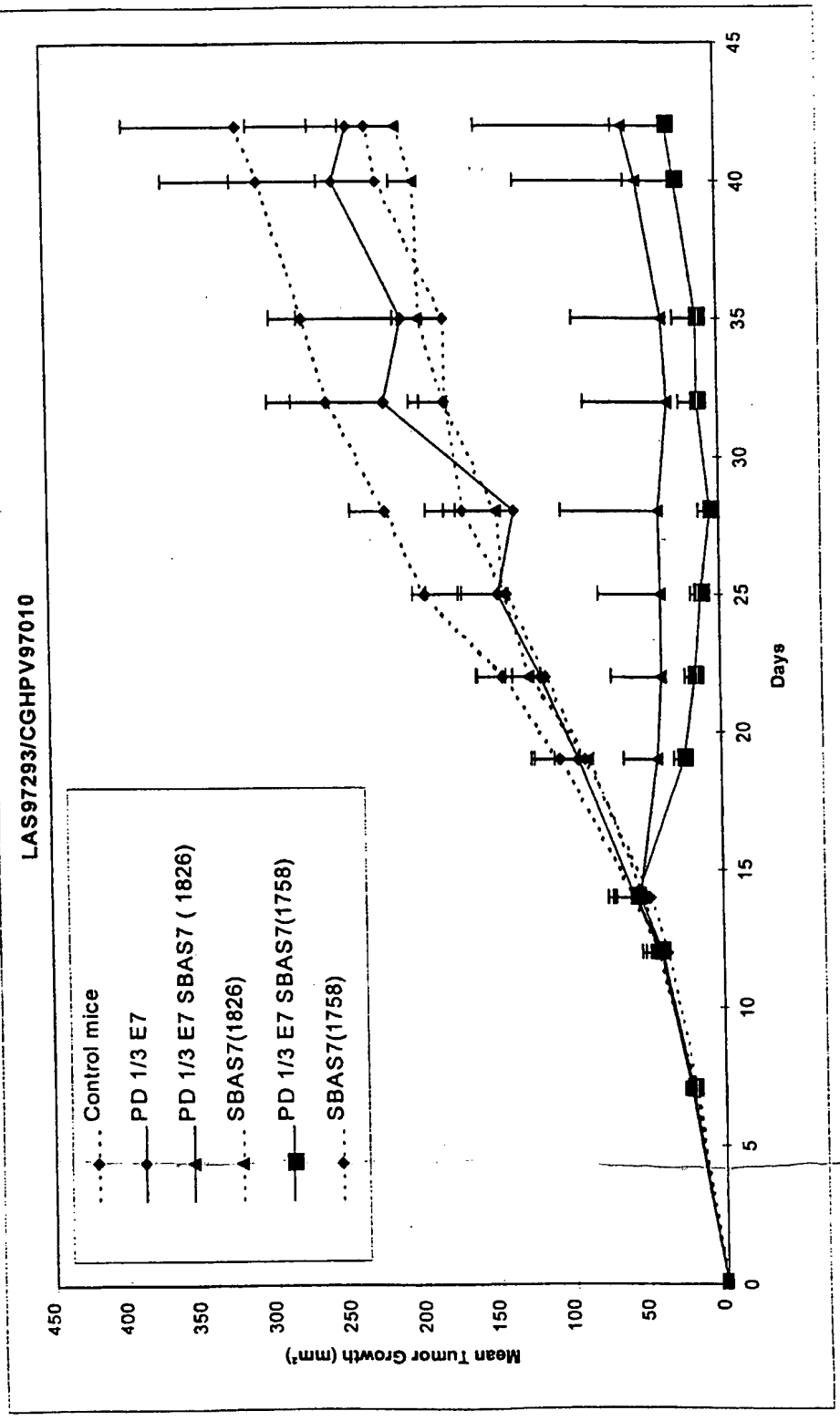
Fig. 2


LAS97293/CGHPV97010
Mean Tumor Growth (mm²)
Days
Control mice
PD 1/3 E7
PD 1/3 E7 SBAS7 (1826)
SBAS7(1826)
PD 1/3 E7 SBAS7(1758)
SBAS7(1758)
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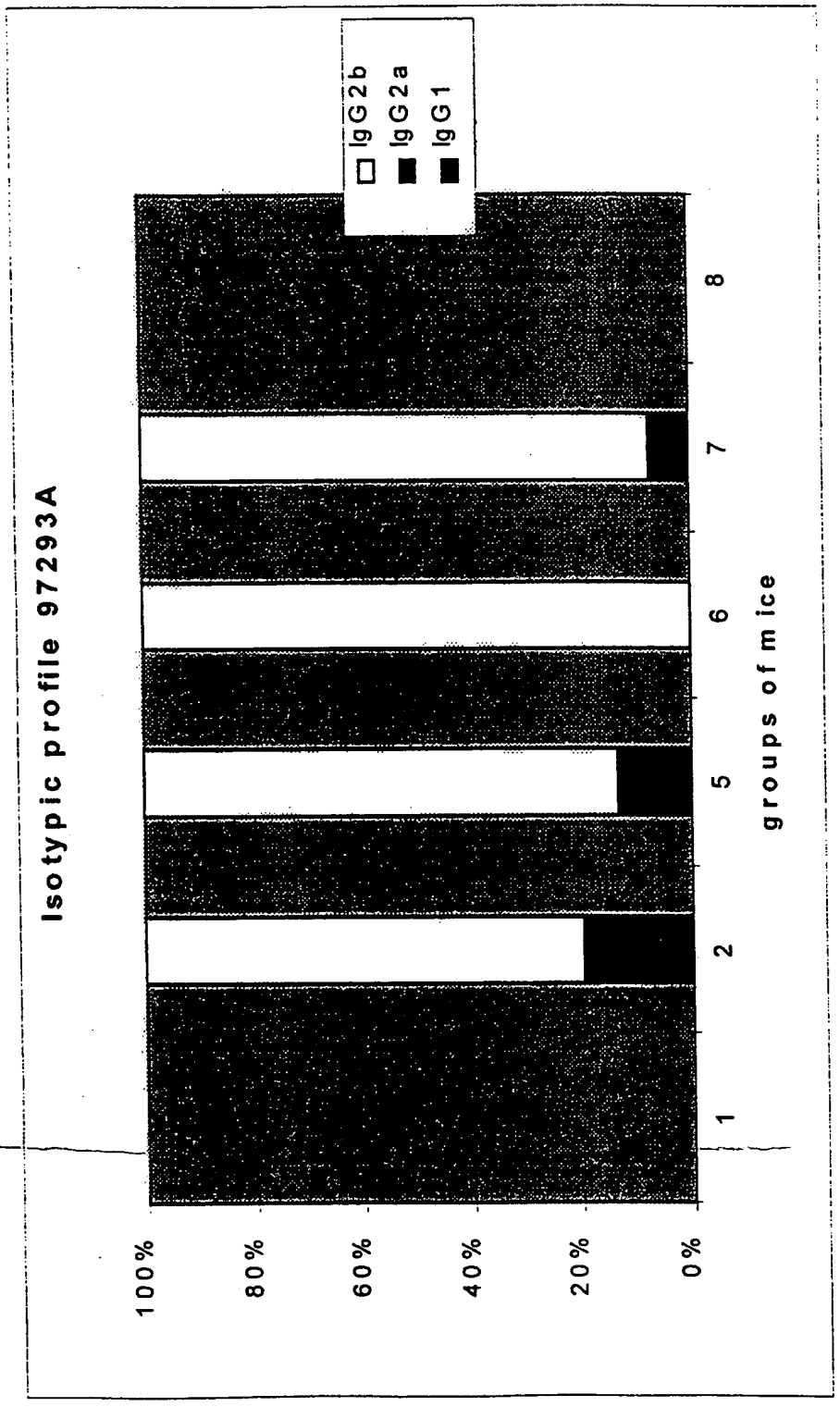
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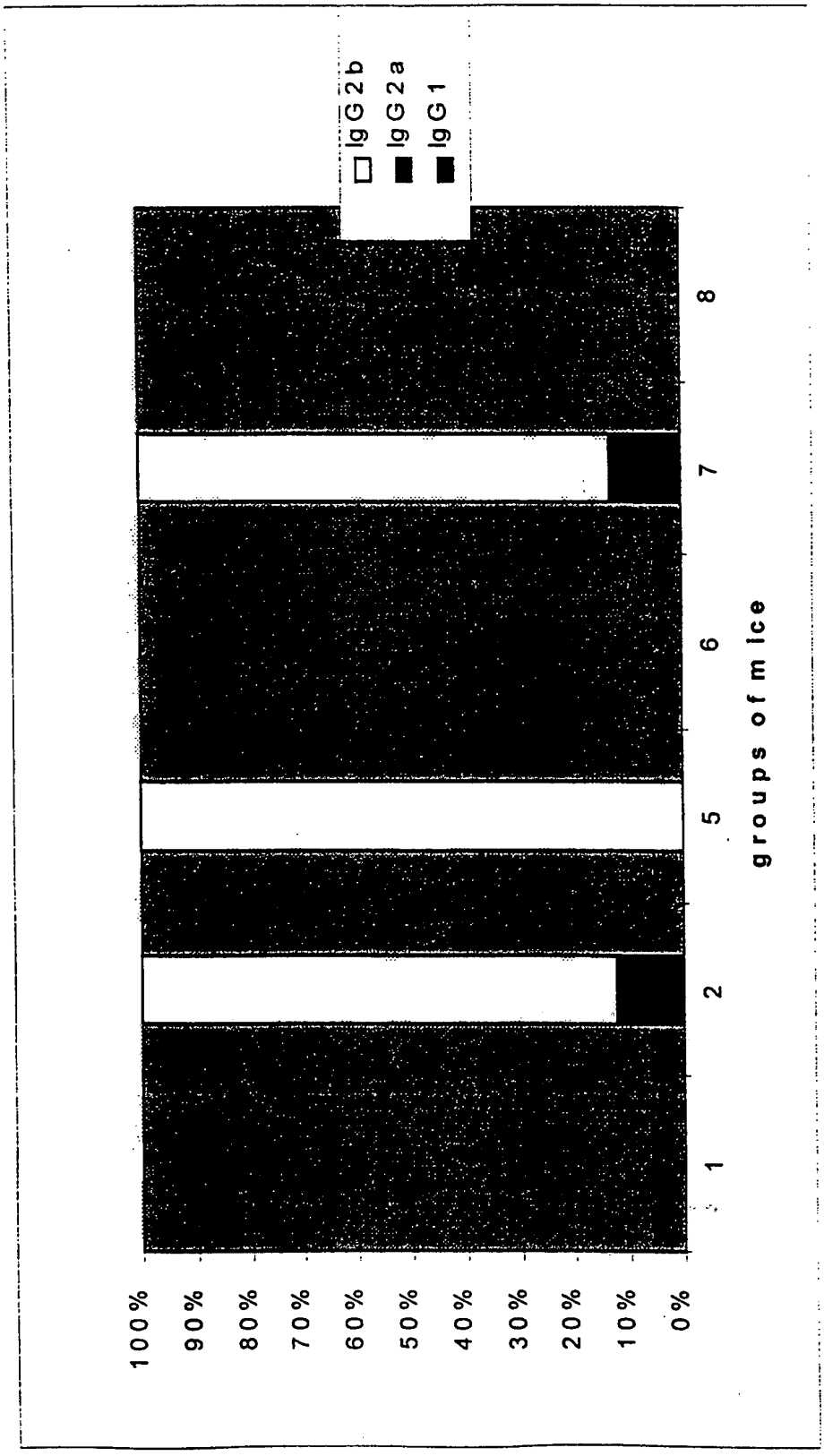
7
8
groups of mice

Fig. 3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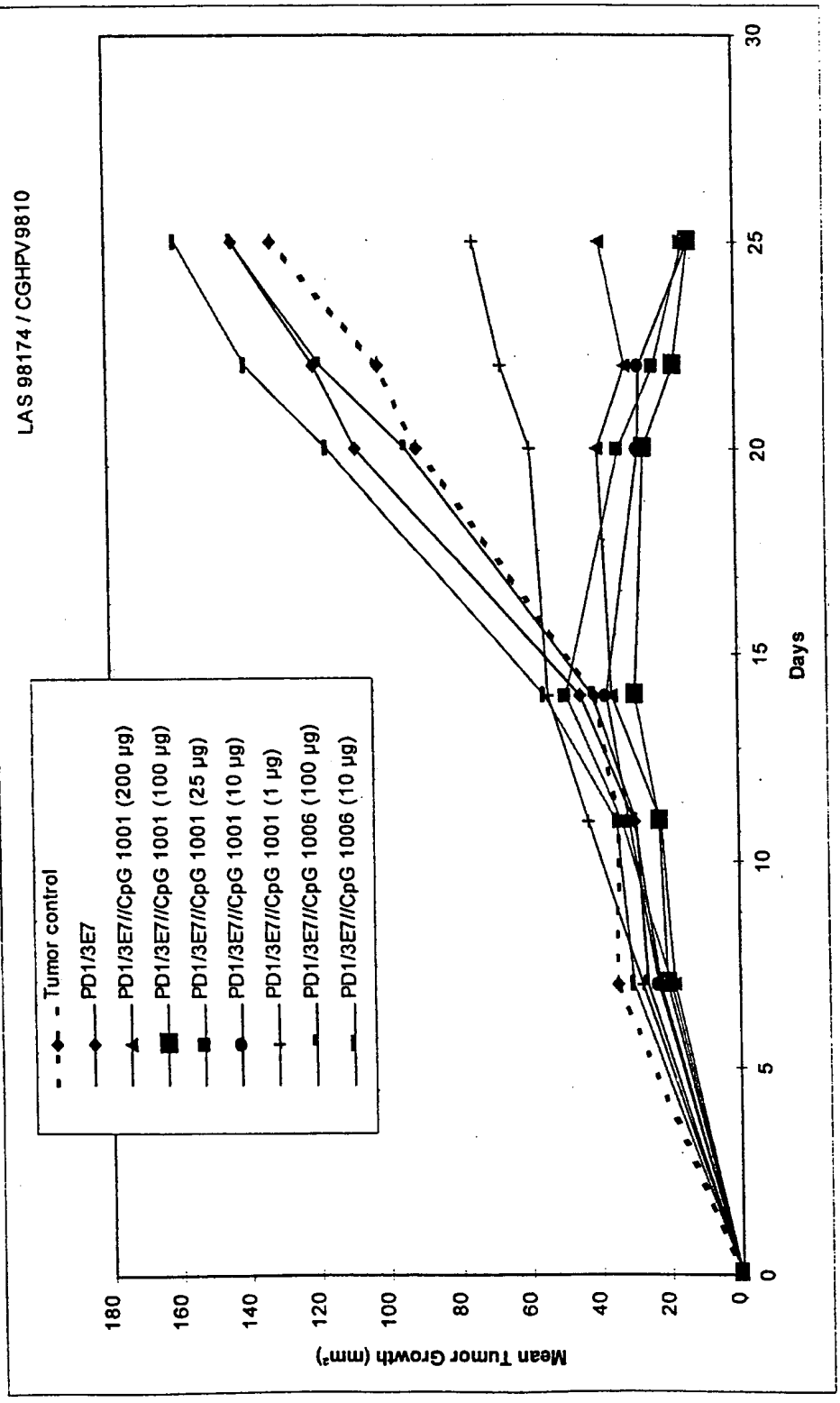
7
8
groups of mice

Fig. 4

Fig. 5


LAS 98174 / CGHPV9810
Mean Tumor Growth (mm²)
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80
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140
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10
15
20
25
30
Tumor control
PD1/3E7
PD1/3E7//CpG 1001 (200 µg)
PD1/3E7//CpG 1001 (100 µg)
PD1/3E7//CpG 1001 (25 µg)
PD1/3E7//CpG 1001 (10 µg)
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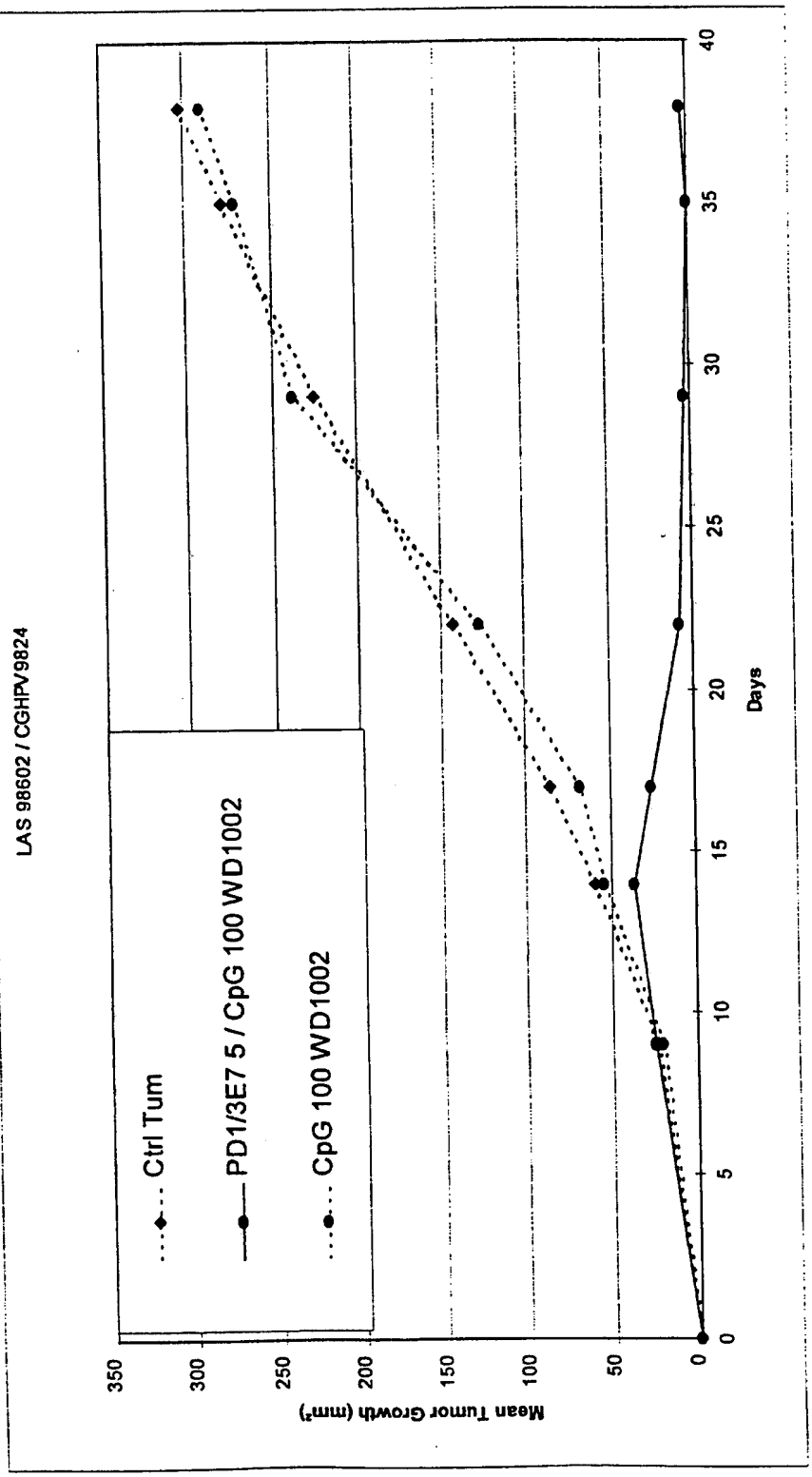
Fig. 6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CpG 100 WD1002
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5
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15
20
25
30
35
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SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT: BRUCK, CLAUDINE

(ii) TITLE OF THE INVENTION: VACCINE

(iii) NUMBER OF SEQUENCES: 23

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: SmithKline Beecham
(B) STREET: 2 New Horizons Court, Great West Road, B
(C) CITY: Middx
(D) STATE:
(E) COUNTRY: UK
(F) ZIP: TW8 9EP

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: DOS
(D) SOFTWARE: FastSEQ for Windows Version 2.0

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Dalton, Marcus J
(B) REGISTRATION NUMBER:
(C) REFERENCE/DOCKET NUMBER: B45124

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 0181 9756348
(B) TELEFAX: 0181 9756177
(C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 220 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
Protein D 1/3 E7 his

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
Protein D 1/3 E7 his

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AAGCCACTGT GTCCTGAAGA AAAGCAAAGA CATCTGGACA AAAAGCAAAG ATTCCATAAT 720
ATAAGGGGTC GGTGGACCGG TCGATGTATG TCTTGTTGCA GATCATCAAG AACACGTAGA 780
GAAACCCAGC TGACTAGTGG CCACCATCAC CATCACCATT AA 822

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 274 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
Protein D 1/3 E6 His/HPV 16

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Asp Pro Ser Ser His Ser Ser Asn Met Ala Asn Thr Gln Met Lys
1 5 10 15
Ser Asp Lys Ile Ile Ile Ala His Arg Gly Ala Ser Gly Tyr Leu Pro
20 25 30
Glu His Thr Leu Glu Ser Lys Ala Leu Ala Phe Ala Gln Gln Ala Asp
35 40 45
Tyr Leu Glu Gln Asp Leu Ala Met Thr Lys Asp Gly Arg Leu Val Val
50 55 60
Ile His Asp His Phe Leu Asp Gly Leu Thr Asp Val Ala Lys Lys Phe
65 70 75 80
Pro His Arg His Arg Lys Asp Gly Arg Tyr Tyr Val Ile Asp Phe Thr
85 90 95
Leu Lys Glu Ile Gln Ser Leu Glu Met Thr Glu Asn Phe Glu Thr Met
100 105 110
Ala Met Phe Gln Asp Pro Gln Glu Arg Pro Arg Lys Leu Pro Gln Leu
115 120 125
Cys Thr Glu Leu Gln Thr Thr Ile His Asp Ile Ile Leu Glu Cys Val
130 135 140
Tyr Cys Lys Gln Gln Leu Leu Arg Arg Glu Val Tyr Asp Phe Ala Phe
145 150 155 160
Arg Asp Leu Cys Ile Val Tyr Arg Asp Gly Asn Pro Tyr Ala Val Cys
165 170 175
Asp Lys Cys Leu Lys Phe Tyr Ser Lys Ile Ser Glu Tyr Arg His Tyr

```
                180                 185                 190
Cys Tyr Ser Leu Tyr Gly Thr Thr Leu Glu Gln Gln Tyr Asn Lys Pro
        195                 200                 205
Leu Cys Asp Leu Leu Ile Arg Cys Ile Asn Cys Gln Lys Pro Leu Cys
    210                 215                 220
Pro Glu Glu Lys Gln Arg His Leu Asp Lys Lys Gln Arg Phe His Asn
225                 230                 235                 240
Ile Arg Gly Arg Trp Thr Gly Arg Cys Met Ser Cys Cys Arg Ser Ser
                245                 250                 255
Arg Thr Arg Arg Glu Thr Gln Leu Thr Ser Gly His His His His His
            260                 265                 270
His
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1116 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
Protein D 1/3 E6/E7/ HPV16

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
ATGGATCCAA GCAGCCATTC ATCAAATATG GCGAATACCC AAATGAAATC AGACAAAATC
60
ATTATTGCTC ACCGTGGTGC TAGCGGTTAT TTACCAGAGC ATACGTTAGA ATCTAAAGCA
120
CTTGCGTTTG CACAACAGGC TGATTATTTA GAGCAAGATT TAGCAATGAC TAAGGATGGT
180
CGTTTAGTGG TTATTCACGA TCACTTTTTA GATGGCTTGA CTGATGTTGC GAAAAAATTC
240
CCACATCGTC ATCGTAAAGA TGGCCGTTAC TATGTCATCG ACTTTACCTT AAAAGAAATT
300
CAAAGTTTAG AAATGACAGA AAACTTTGAA ACCATGGCCA TGTTTCAGGA CCCACAGGAG
360
CGACCCAGAA AGTTACCACA GTTATGCACA GAGCTGCAAA CAACTATACA TGATATAATA
420
TTAGAATGTG TGTACTGCAA GCAACAGTTA CTGCGACGTG AGGTATATGA CTTTGCTTTT
480
CGGGATTTAT GCATAGTATA TAGAGATGGG AATCCATATG CTGTATGTGA TAAATGTTTA
540
AAGTTTTATT CTAAAATTAG TGAGTATAGA CATTATTGTT ATAGTTTGTA TGGAACAACA
600
TTAGAACAGC AATACAACAA ACCGTTGTGT GATTTGTTAA TTAGGTGTAT TAACTGTCAA
660
AAGCCACTGT GTCCTGAAGA AAAGCAAAGA CATCTGGACA AAAAGCAAAG ATTCCATAAT
720
ATAAGGGGTC GGTGGACCGG TCGATGTATG TCTTGTTGCA GATCATCAAG AACACGTAGA
780
GAAACCCAGC TGATGCATGG AGATACACCT ACATTGCATG AATATATGTT AGATTTGCAA
840
CCAGAGACAA CTGATCTCTA CTGTTATGAG CAATTAAATG ACAGCTCAGA GGAGGAGGAT
900
GAAATAGATG GTCCAGCTGG ACAAGCAGAA CCGGACAGAG CCCATTACAA TATTGTAACC
960
TTTTGTTGCA AGTGTGACTC TACGCTTCGG TTGTGCGTAC AAAGCACACA CGTAGACATT
1020
CGTACTTTGG AAGACCTGTT AATGGGCACA CTAGGAATTG TGTGCCCCAT CTGTTCTCAG
1080
AAACCAACTA GTGGCCACCA TCACCATCAC CATTAA
1116
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 372 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
Protein D 1/3 E6/E7/ HPV16

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
Met Asp Pro Ser Ser His Ser Ser Asn Met Ala Asn Thr Gln Met Lys
 1               5                  10                  15
Ser Asp Lys Ile Ile Ile Ala His Arg Gly Ala Ser Gly Tyr Leu Pro
            20                  25                  30
Glu His Thr Leu Glu Ser Lys Ala Leu Ala Phe Ala Gln Gln Ala Asp
        35                  40                  45
Tyr Leu Glu Gln Asp Leu Ala Met Thr Lys Asp Gly Arg Leu Val Val
    50                  55                  60
Ile His Asp His Phe Leu Asp Gly Leu Thr Asp Val Ala Lys Lys Phe
65                  70                  75                  80
Pro His Arg His Arg Lys Asp Gly Arg Tyr Tyr Val Ile Asp Phe Thr
                85                  90                  95
Leu Lys Glu Ile Gln Ser Leu Glu Met Thr Glu Asn Phe Glu Thr Met
            100                 105                 110
Ala Met Phe Gln Asp Pro Gln Glu Arg Pro Arg Lys Leu Pro Gln Leu
        115                 120                 125
Cys Thr Glu Leu Gln Thr Thr Ile His Asp Ile Ile Leu Glu Cys Val
    130                 135                 140
Tyr Cys Lys Gln Gln Leu Leu Arg Arg Glu Val Tyr Asp Phe Ala Phe
145                 150                 155                 160
Arg Asp Leu Cys Ile Val Tyr Arg Asp Gly Asn Pro Tyr Ala Val Cys
                165                 170                 175
Asp Lys Cys Leu Lys Phe Tyr Ser Lys Ile Ser Glu Tyr Arg His Tyr
            180                 185                 190
Cys Tyr Ser Leu Tyr Gly Thr Thr Leu Glu Gln Gln Tyr Asn Lys Pro
        195                 200                 205
Leu Cys Asp Leu Leu Ile Arg Cys Ile Asn Cys Gln Lys Pro Leu Cys
    210                 215                 220
Pro Glu Glu Lys Gln Arg His Leu Asp Lys Lys Gln Arg Phe His Asn
225                 230                 235                 240
Ile Arg Gly Arg Trp Thr Gly Arg Cys Met Ser Cys Cys Arg Ser Ser
                245                 250                 255
Arg Thr Arg Arg Glu Thr Gln Leu Met His Gly Asp Thr Pro Thr Leu
            260                 265                 270
His Glu Tyr Met Leu Asp Leu Gln Pro Glu Thr Thr Asp Leu Tyr Cys
        275                 280                 285
Tyr Glu Gln Leu Asn Asp Ser Ser Glu Glu Glu Asp Glu Ile Asp Gly
    290                 295                 300
Pro Ala Gly Gln Ala Glu Pro Asp Arg Ala His Tyr Asn Ile Val Thr
305                 310                 315                 320
Phe Cys Cys Lys Cys Asp Ser Thr Leu Arg Leu Cys Val Gln Ser Thr
                325                 330                 335
His Val Asp Ile Arg Thr Leu Glu Asp Leu Leu Met Gly Thr Leu Gly
            340                 345                 350
Ile Val Cys Pro Ile Cys Ser Gln Lys Pro Thr Ser Gly His His His
        355                 360                 365
His His His
    370
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 663 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
Protein D 1/3 E7 mutated HPV 16

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ATGGATCCAA GCAGCCATTC ATCAAATATG GCGAATACCC AAATGAAATC AGACAAAATC 60
ATTATTGCTC ACCGTGGTGC TAGCGGTTAT TTACCAGAGC ATACGTTAGA ATCTAAAGCA 120
CTTGCGTTTG CACAACAGGC TGATTATTTA GAGCAAGATT TAGCAATGAC TAAGGATGGT 180
CGTTTAGTGG TTATTCACGA TCACTTTTTA GATGGCTTGA CTGATGTTGC GAAAAAATTC 240
CCACATCGTC ATCGTAAAGA TGGCCGTTAC TATGTCATCG ACTTTACCTT AAAAGAAATT 300
CAAAGTTTAG AAATGACAGA AACTTTGAA ACCATGGCCA TGCATGGAGA TACACCTACA 360
TTGCATGAAT ATATGTTAGA TTTGCAACCA GAGACAACTG ATCTCTACGG TTATCAGCAA 420
TTAAATGACA GCTCAGAGGA GGAGGATGAA ATAGATGGTC CAGCTGGACA AGCAGAACCG 480
GACAGAGCCC ATTACAATAT TGTAACCTTT TGTTGCAAGT GTGACTCTAC GCTTCGGTTG 540
TGCGTACAAA GCACACACGT AGACATTCGT ACTTTGGAAG ACCTGTTAAT GGGCACACTA 600
GGAATTGTGT GCCCCATCTG TTCTCAGAAA CCAACTAGTG GCCACCATCA CCATCACCAT 660
TAA
663

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 220 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

Protein D 1/3 E7 mutated HPV 16
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Met Asp Pro Ser Ser His Ser Ser Asn Met Ala Asn Thr Gln Met Lys
1 5 10 15
Ser Asp Lys Ile Ile Ile Ala His Arg Gly Ala Ser Gly Tyr Leu Pro
20 25 30
Glu His Thr Leu Glu Ser Lys Ala Leu Ala Phe Ala Gln Gln Ala Asp
35 40 45
Tyr Leu Glu Gln Asp Leu Ala Met Thr Lys Asp Gly Arg Leu Val Val
50 55 60
Ile His Asp His Phe Leu Asp Gly Leu Thr Asp Val Ala Lys Lys Phe
65 70 75 80
Pro His Arg His Arg Lys Asp Gly Arg Tyr Tyr Val Ile Asp Phe Thr
85 90 95
Leu Lys Glu Ile Gln Ser Leu Glu Met Thr Glu Asn Phe Glu Thr Met
100 105 110
Ala Met His Gly Asp Thr Pro Thr Leu His Glu Tyr Met Leu Asp Leu
115 120 125
Gln Pro Glu Thr Thr Asp Leu Tyr Gly Tyr Gln Gln Leu Asn Asp Ser
130 135 140
Ser Glu Glu Glu Asp Glu Ile Asp Gly Pro Ala Gly Gln Ala Glu Pro
145 150 155 160
Asp Arg Ala His Tyr Asn Ile Val Thr Phe Cys Cys Lys Cys Asp Ser
165 170 175
Thr Leu Arg Leu Cys Val Gln Ser Thr His Val Asp Ile Arg Thr Leu
180 185 190
Glu Asp Leu Leu Met Gly Thr Leu Gly Ile Val Cys Pro Ile Cys Ser
195 200 205
Gln Lys Pro Thr Ser Gly His His His His His His

210 215 220

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 879 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
CLYTA E6 His HPV 16

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ATGAAAGGGG GAATTGTACA TTCAGACGGC TCTTATCCAA AAGACAAGTT TGAGAAAATC 60
AATGGCACTT GGTACTACTT TGACAGTTCA GGCTATATGC TTGCAGACCG CTGGAGGAAG 120
CACACAGACG GCAACTGGTA CTGGTTCGAC AACTCAGGCG AAATGGCTAC AGGCTGGAAG 180
AAAATCGCTG ATAAGTGGTA CTATTTCAAC GAAGAAGGTG CCATGAAGAC AGGCTGGGTC 240
AAGTACAAGG ACACTTGGTA CTACTTAGAC GCTAAAGAAG GCGCCATGGT ATCAAATGCC 300
TTTATCCAGT CAGCGGACGG AACAGGCTGG TACTACCTCA AACCAGACGG AACACTGGCA 360
GACAGGCCAG AATTGGCCAG CATGCTGGAC ATGGCCATGT TTCAGGACCC ACAGGAGCGA 420
CCCAGAAAGT TACCACAGTT ATGCACAGAG CTGCAAACAA CTATACATGA TATAATATTA 480
GAATGTGTGT ACTGCAAGCA ACAGTTACTG CGACGTGAGG TATATGACTT TGCTTTTCGG 540
GATTTATGCA TAGTATATAG AGATGGGAAT CCATATGCTG TATGTGATAA ATGTTTAAAG 600
TTTTATTCTA AAATTAGTGA GTATAGACAT TATTGTTATA GTTTGTATGG AACAACATTA 660
GAACAGCAAT ACAACAAACC GTTGTGTGAT TTGTTAATTA GGTGTATTAA CTGTCAAAAG 720
CCACTGTGTC CTGAAGAAAA GCAAAGACAT CTGGACAAAA AGCAAAGATT CCATAATATA 780
AGGGGTCGGT GGACCGGTCG ATGTATGTCT TGTTGCAGAT CATCAAGAAC ACGTAGAGAA 840
ACCCAGCTGA CTAGTGGCCA CCATCACCAT CACCATTAA 879

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 293 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
CLYTA E6 His HPV 16

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Met Lys Gly Gly Ile Val His Ser Asp Gly Ser Tyr Pro Lys Asp Lys
1 5 10 15
Phe Glu Lys Ile Asn Gly Thr Trp Tyr Tyr Phe Asp Ser Ser Gly Tyr
20 25 30
Met Leu Ala Asp Arg Trp Arg Lys His Thr Asp Gly Asn Trp Tyr Trp
35 40 45
Phe Asp Asn Ser Gly Glu Met Ala Thr Gly Trp Lys Lys Ile Ala Asp
50 55 60
Lys Trp Tyr Tyr Phe Asn Glu Glu Gly Ala Met Lys Thr Gly Trp Val
65 70 75 80
Lys Tyr Lys Asp Thr Trp Tyr Tyr Leu Asp Ala Lys Glu Gly Ala Met

```
                    85                  90                  95
Val Ser Asn Ala Phe Ile Gln Ser Ala Asp Gly Thr Gly Trp Tyr Tyr
                100                 105                 110
Leu Lys Pro Asp Gly Thr Leu Ala Asp Arg Pro Glu Leu Ala Ser Met
            115                 120                 125
Leu Asp Met Ala Met Phe Gln Asp Pro Gln Glu Arg Pro Arg Lys Leu
        130                 135                 140
Pro Gln Leu Cys Thr Glu Leu Gln Thr Thr Ile His Asp Ile Ile Leu
145                 150                 155                 160
Glu Cys Val Tyr Cys Lys Gln Gln Leu Leu Arg Arg Glu Val Tyr Asp
                165                 170                 175
Phe Ala Phe Arg Asp Leu Cys Ile Val Tyr Arg Asp Gly Asn Pro Tyr
            180                 185                 190
Ala Val Cys Asp Lys Cys Leu Lys Phe Tyr Ser Lys Ile Ser Glu Tyr
        195                 200                 205
Arg His Tyr Cys Tyr Ser Leu Tyr Gly Thr Thr Leu Glu Gln Gln Tyr
    210                 215                 220
Asn Lys Pro Leu Cys Asp Leu Leu Ile Arg Cys Ile Asn Cys Gln Lys
225                 230                 235                 240
Pro Leu Cys Pro Glu Glu Lys Gln Arg His Leu Asp Lys Lys Gln Arg
                245                 250                 255
Phe His Asn Ile Arg Gly Arg Trp Thr Gly Arg Cys Met Ser Cys Cys
            260                 265                 270
Arg Ser Ser Arg Thr Arg Arg Glu Thr Gln Leu Thr Ser Gly His His
        275                 280                 285
His His His His
    290
```

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 720 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
CLYTA E7 HIS HPV 16

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```
ATGAAAGGGG GAATTGTACA TTCAGACGGC TCTTATCCAA AAGACAAGTT TGAGAAAATC
60
AATGGCACTT GGTACTACTT TGACAGTTCA GGCTATATGC TTGCAGACCG CTGGAGGAAG
120
CACACAGACG GCAACTGGTA CTGGTTCGAC AACTCAGGCG AAATGGCTAC AGGCTGGAAG
180
AAAATCGCTG ATAAGTGGTA CTATTTCAAC GAAGAAGGTG CCATGAAGAC AGGCTGGGTC
240
AAGTACAAGG ACACTTGGTA CTACTTAGAC GCTAAAGAAG GCGCCATGGT ATCAAATGCC
300
TTTATCCAGT CAGCGGACGG AACAGGCTGG TACTACCTCA AACCAGACGG AACACTGGCA
360
GACAGGCCAG AATTGGCCAG CATGCTGGAC ATGGCCATGC ATGGAGATAC ACCTACATTG
420
CATGAATATA TGTTAGATTT GCAACCAGAG ACAACTGATC TCTACTGTTA TGAGCAATTA
480
AATGACAGCT CAGAGGAGGA GGATGAAATA GATGGTCCAG CTGGACAAGC AGAACCGGAC
540
AGAGCCCATT ACAATATTGT AACCTTTTGT TGCAAGTGTG ACTCTACGCT TCGGTTGTGC
600
GTACAAAGCA CACACGTAGA CATTCGTACT TTGGAAGACC TGTTAATGGG CACACTAGGA
660
ATTGTGTGCC CCATCTGTTC TCAGAAACCA ACTAGTGGCC ACCATCACCA TCACCATTAA
720
```

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 240 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
CLYTA E7 HIS HPV 16

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```
Met Lys Gly Gly Ile Val His Ser Asp Gly Ser Tyr Pro Lys Asp Lys
 1               5                  10                  15
Phe Glu Lys Ile Asn Gly Thr Trp Tyr Tyr Phe Asp Ser Ser Gly Tyr
            20                  25                  30
Met Leu Ala Asp Arg Trp Arg Lys His Thr Asp Gly Asn Trp Tyr Trp
        35                  40                  45
Phe Asp Asn Ser Gly Glu Met Ala Thr Gly Trp Lys Lys Ile Ala Asp
    50                  55                  60
Lys Trp Tyr Tyr Phe Asn Glu Glu Gly Ala Met Lys Thr Gly Trp Val
65                  70                  75                  80
Lys Tyr Lys Asp Thr Trp Tyr Tyr Leu Asp Ala Lys Glu Gly Ala Met
                85                  90                  95
Val Ser Asn Ala Phe Ile Gln Ser Ala Asp Gly Thr Gly Trp Tyr Tyr
            100                 105                 110
Leu Lys Pro Asp Gly Thr Leu Ala Asp Arg Pro Glu Leu Ala Ser Met
        115                 120                 125
Leu Asp Met Ala Met His Gly Asp Thr Pro Thr Leu His Glu Tyr Met
    130                 135                 140
Leu Asp Leu Gln Pro Glu Thr Thr Asp Leu Tyr Cys Tyr Glu Gln Leu
145                 150                 155                 160
Asn Asp Ser Ser Glu Glu Glu Asp Glu Ile Asp Gly Pro Ala Gly Gln
                165                 170                 175
Ala Glu Pro Asp Arg Ala His Tyr Asn Ile Val Thr Phe Cys Cys Lys
            180                 185                 190
Cys Asp Ser Thr Leu Arg Leu Cys Val Gln Ser Thr His Val Asp Ile
        195                 200                 205
Arg Thr Leu Glu Asp Leu Leu Met Gly Thr Leu Gly Ile Val Cys Pro
    210                 215                 220
Ile Cys Ser Gln Lys Pro Thr Ser Gly His His His His His His
225                 230                 235
```

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1173 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
CLYTA E6E7 His HPV16

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```
ATGAAAGGGG GAATTGTACA TTCAGACGGC TCTTATCCAA AAGACAAGTT TGAGAAAATC
60
AATGGCACTT GGTACTACTT TGACAGTTCA GGCTATATGC TTGCAGACCG CTGGAGGAAG
120
CACACAGACG GCAACTGGTA CTGGTTCGAC AACTCAGGCG AAATGGCTAC AGGCTGGAAG
180
AAAATCGCTG ATAAGTGGTA CTATTTCAAC GAAGAAGGTG CCATGAAGAC AGGCTGGGTC
240
AAGTACAAGG ACACTTGGTA CTACTTAGAC GCTAAAGAAG GCGCCATGGT ATCAAATGCC
300
TTTATCCAGT CAGCGGACGG AACAGGCTGG TACTACCTCA AACCAGACGG AACACTGGCA
360
GACAGGCCAG AATTGGCCAG CATGCTGGAC ATGGCCATGT TTCAGGACCC ACAGGAGCGA
420
```

CCCAGAAAGT TACCACAGTT ATGCACAGAG CTGCAAACAA CTATACATGA TATAATATTA
480
GAATGTGTGT ACTGCAAGCA ACAGTTACTG CGACGTGAGG TATATGACTT TGCTTTTCGG
540
GATTTATGCA TAGTATATAG AGATGGGAAT CCATATGCTG TATGTGATAA ATGTTTAAAG
600
TTTTATTCTA AAATTAGTGA GTATAGACAT TATTGTTATA GTTTGTATGG AACAACATTA
660
GAACAGCAAT ACAACAAACC GTTGTGTGAT TTGTTAATTA GGTGTATTAA CTGTCAAAAG
720
CCACTGTGTC CTGAAGAAAA GCAAAGACAT CTGGACAAAA AGCAAAGATT CCATAATATA
780
AGGGGTCGGT GGACCGGTCG ATGTATGTCT TGTTGCAGAT CATCAAGAAC ACGTAGAGAA
840
ACCCAGCTGA TGCATGGAGA TACACCTACA TTGCATGAAT ATATGTTAGA TTTGCAACCA
900
GAGACAACTG ATCTCTACTG TTATGAGCAA TTAAATGACA GCTCAGAGGA GGAGGATGAA
960
ATAGATGGTC CAGCTGGACA AGCAGAACCG GACAGAGCCC ATTACAATAT TGTAACCTTT
1020
TGTTGCAAGT GTGACTCTAC GCTTCGGTTG TGCGTACAAA GCACACACGT AGACATTCGT
1080
ACTTTGGAAG ACCTGTTAAT GGGCACACTA GGAATTGTGT GCCCCATCTG TTCTCAGAAA
1140
CCAACTAGTG GCCACCATCA CCATCACCAT TAA
1173

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 391 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
CLYTA E6E7 His HPV16

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Met Lys Gly Gly Ile Val His Ser Asp Gly Ser Tyr Pro Lys Asp Lys
1 5 10 15
Phe Glu Lys Ile Asn Gly Thr Trp Tyr Tyr Phe Asp Ser Ser Gly Tyr
20 25 30
Met Leu Ala Asp Arg Trp Arg Lys His Thr Asp Gly Asn Trp Tyr Trp
35 40 45
Phe Asp Asn Ser Gly Glu Met Ala Thr Gly Trp Lys Lys Ile Ala Asp
50 55 60
Lys Trp Tyr Tyr Phe Asn Glu Glu Gly Ala Met Lys Thr Gly Trp Val
65 70 75 80
Lys Tyr Lys Asp Thr Trp Tyr Tyr Leu Asp Ala Lys Glu Gly Ala Met
85 90 95
Val Ser Asn Ala Phe Ile Gln Ser Ala Asp Gly Thr Gly Trp Tyr Tyr
100 105 110
Leu Lys Pro Asp Gly Thr Leu Ala Asp Arg Pro Glu Leu Ala Ser Met
115 120 125
Leu Asp Met Ala Met Phe Gln Asp Pro Gln Glu Arg Pro Arg Lys Leu
130 135 140
Pro Gln Leu Cys Thr Glu Leu Gln Thr Thr Ile His Asp Ile Ile Leu
145 150 155 160
Glu Cys Val Tyr Cys Lys Gln Gln Leu Leu Arg Arg Glu Val Tyr Asp
165 170 175
Phe Ala Phe Arg Asp Leu Cys Ile Val Tyr Arg Asp Gly Asn Pro Tyr
180 185 190
Ala Val Cys Asp Lys Cys Leu Lys Phe Tyr Ser Lys Ile Ser Glu Tyr
195 200 205
Arg His Tyr Cys Tyr Ser Leu Tyr Gly Thr Thr Leu Glu Gln Gln Tyr
210 215 220

```
Asn Lys Pro Leu Cys Asp Leu Leu Ile Arg Cys Ile Asn Cys Gln Lys
225                 230                 235                 240
Pro Leu Cys Pro Glu Glu Lys Gln Arg His Leu Asp Lys Lys Gln Arg
                245                 250                 255
Phe His Asn Ile Arg Gly Arg Trp Thr Gly Arg Cys Met Ser Cys Cys
            260                 265                 270
Arg Ser Ser Arg Thr Arg Arg Glu Thr Gln Leu Met His Gly Asp Thr
        275                 280                 285
Pro Thr Leu His Glu Tyr Met Leu Asp Leu Gln Pro Glu Thr Thr Asp
    290                 295                 300
Leu Tyr Cys Tyr Glu Gln Leu Asn Asp Ser Ser Glu Glu Glu Asp Glu
305                 310                 315                 320
Ile Asp Gly Pro Ala Gly Gln Ala Glu Pro Asp Arg Ala His Tyr Asn
                325                 330                 335
Ile Val Thr Phe Cys Cys Lys Cys Asp Ser Thr Leu Arg Leu Cys Val
            340                 345                 350
Gln Ser Thr His Val Asp Ile Arg Thr Leu Glu Asp Leu Leu Met Gly
        355                 360                 365
Thr Leu Gly Ile Val Cys Pro Ile Cys Ser Gln Lys Pro Thr Ser Gly
    370                 375                 380
His His His His His His
385                 390
```

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 684 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
Protein D 1/3 E7 his HPV 18

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

```
ATGGATCCAA GCAGCCATTC ATCAAATATG GCGAATACCC AAATGAAATC AGACAAAATC
60
ATTATTGCTC ACCGTGGTGC TAGCGGTTAT TTACCAGAGC ATACGTTAGA ATCTAAAGCA
120
CTTGCGTTTG CACAACAGGC TGATTATTTA GAGCAAGATT TAGCAATGAC TAAGGATGGT
180
CGTTTAGTGG TTATTCACGA TCACTTTTTA GATGGCTTGA CTGATGTTGC GAAAAAATTC
240
CCACATCGTC ATCGTAAAGA TGGCCGTTAC TATGTCATCG ACTTTACCTT AAAAGAAATT
300
CAAAGTTTAG AAATGACAGA AAACTTTGAA ACCATGGCCA TGCATGGACC TAAGGCAACA
360
TTGCAAGACA TTGTATTGCA TTTAGAGCCC CAAAATGAAA TTCCGGTTGA CCTTCTATGT
420
CACGAGCAAT TAAGCGACTC AGAGGAAGAA AACGATGAAA TAGATGAAGT TAATCATCAA
480
CATTTACCAG CCCGACGAGC CGAACCACAA CGTCACACAA TGTTGTGTAT GTGTTGTAAG
540
TGTGAAGCCA GAATTGAGCT AGTAGTAGAA AGCTCAGCAG ACGACCTTCG AGCATTCCAG
600
CAGCTGTTTC TGAACACCCT GTCCTTTGTG TGTCCGTGGT GTGCATCCCA GCAGACTAGT
660
GGCCACCATC ACCATCACCA TTAA
684
```

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 228 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear
Protein D 1/3 E7 his HPV 18

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

```
Met Asp Pro Ser Ser His Ser Ser Asn Met Ala Asn Thr Gln Met Lys
 1               5                  10                  15
Ser Asp Lys Ile Ile Ile Ala His Arg Gly Ala Ser Gly Tyr Leu Pro
            20                  25                  30
Glu His Thr Leu Glu Ser Lys Ala Leu Ala Phe Ala Gln Gln Ala Asp
        35                  40                  45
Tyr Leu Glu Gln Asp Leu Ala Met Thr Lys Asp Gly Arg Leu Val Val
    50                  55                  60
Ile His Asp His Phe Leu Asp Gly Leu Thr Asp Val Ala Lys Lys Phe
65                  70                  75                  80
Pro His Arg His Arg Lys Asp Gly Arg Tyr Tyr Val Ile Asp Phe Thr
                85                  90                  95
Leu Lys Glu Ile Gln Ser Leu Glu Met Thr Glu Asn Phe Glu Thr Met
            100                 105                 110
Ala Met His Gly Pro Lys Ala Thr Leu Gln Asp Ile Val Leu His Leu
        115                 120                 125
Glu Pro Gln Asn Glu Ile Pro Val Asp Leu Leu Cys His Glu Gln Leu
    130                 135                 140
Ser Asp Ser Glu Glu Glu Asn Asp Glu Ile Asp Glu Val Asn His Gln
145                 150                 155                 160
His Leu Pro Ala Arg Arg Ala Glu Pro Gln Arg His Thr Met Leu Cys
                165                 170                 175
Met Cys Cys Lys Cys Glu Ala Arg Ile Glu Leu Val Val Glu Ser Ser
            180                 185                 190
Ala Asp Asp Leu Arg Ala Phe Gln Gln Leu Phe Leu Asn Thr Leu Ser
        195                 200                 205
Phe Val Cys Pro Trp Cys Ala Ser Gln Gln Thr Ser Gly His His His
    210                 215                 220
His His His
225
```

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 110 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
Thioredoxin

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

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Met Ser Asp Lys Ile Ile His Leu Thr Asp Asp Ser Phe Asp Thr Asp
 1               5                  10                  15
Val Leu Lys Ala Asp Gly Ala Ile Leu Val Asp Phe Trp Ala Glu Trp
            20                  25                  30
Cys Gly Pro Cys Lys Met Ile Ala Pro Ile Leu Asp Glu Ile Ala Asp
        35                  40                  45
Glu Tyr Gln Gly Lys Leu Thr Val Ala Lys Leu Asn Ile Asp Gln Asn
    50                  55                  60
Pro Gly Thr Ala Pro Lys Tyr Gly Ile Arg Gly Ile Pro Thr Leu Leu
65                  70                  75                  80
Leu Phe Lys Asn Gly Glu Val Ala Ala Thr Lys Val Gly Ala Leu Ser
                85                  90                  95
Lys Gly Gln Leu Lys Glu Phe Leu Asp Ala Asn Leu Ala
            100                 105
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(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 684 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
Protein D 1/3 E7 mutated HPV 18

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

ATGGATCCAA GCAGCCATTC ATCAAATATG GCGAATACCC AAATGAAATC AGACAAAATC
60
ATTATTGCTC ACCGTGGTGC TAGCGGTTAT TTACCAGAGC ATACGTTAGA ATCTAAAGCA
120
CTTGCGTTTG CACAACAGGC TGATTATTTA GAGCAAGATT TAGCAATGAC TAAGGATGGT
180
CGTTTAGTGG TTATTCACGA TCACTTTTTA GATGGCTTGA CTGATGTTGC GAAAAAATTC
240
CCACATCGTC ATCGTAAAGA TGGCCGTTAC TATGTCATCG ACTTTACCTT AAAAGAAATT
300
CAAAGTTTAG AAATGACAGA AAACTTTGAA ACCATGGCCA TGCATGGACC TAAGGCAACA
360
TTGCAAGACA TTGTATTGCA TTTAGAGCCC CAAAATGAAA TTCCGGTTGA CCTTCTAGGT
420
CACCAGCAAT TAAGCGACTC AGAGGAAGAA AACGATGAAA TAGATGGAGT TAATCATCAA
480
CATTTACCAG CCCGACGAGC CGAACCACAA CGTCACACAA TGTTGTGTAT GTGTTGTAAG
540
TGTGAAGCCA GAATTGAGCT AGTAGTAGAA AGCTCAGCAG ACGACCTTCG AGCATTCCAG
600
CAGCTGTTTC TGAACACCCT GTCCTTTGTG TGTCCGTGGT GTGCATCCCA GCAGACTAGT
660
GGCCACCATC ACCATCACCA TTAA
684

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 228 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
Protein D 1/3 E7 mutated HPV 18

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Met Asp Pro Ser Ser His Ser Ser Asn Met Ala Asn Thr Gln Met Lys
1 5 10 15
Ser Asp Lys Ile Ile Ile Ala His Arg Gly Ala Ser Gly Tyr Leu Pro
20 25 30
Glu His Thr Leu Glu Ser Lys Ala Leu Ala Phe Ala Gln Gln Ala Asp
35 40 45
Tyr Leu Glu Gln Asp Leu Ala Met Thr Lys Asp Gly Arg Leu Val Val
50 55 60
Ile His Asp His Phe Leu Asp Gly Leu Thr Asp Val Ala Lys Lys Phe
65 70 75 80
Pro His Arg His Arg Lys Asp Gly Arg Tyr Tyr Val Ile Asp Phe Thr
85 90 95
Leu Lys Glu Ile Gln Ser Leu Glu Met Thr Glu Asn Phe Glu Thr Met
100 105 110
Ala Met His Gly Pro Lys Ala Thr Leu Gln Asp Ile Val Leu His Leu
115 120 125
Glu Pro Gln Asn Glu Ile Pro Val Asp Leu Leu Gly His Gln Gln Leu
130 135 140
Ser Asp Ser Glu Glu Glu Asn Asp Glu Ile Asp Gly Val Asn His Gln
145 150 155 160
His Leu Pro Ala Arg Arg Ala Glu Pro Gln Arg His Thr Met Leu Cys
165 170 175

Met Cys Cys Lys Cys Glu Ala Arg Ile Glu Leu Val Val Glu Ser Ser
180 185 190
Ala Asp Asp Leu Arg Ala Phe Gln Gln Leu Phe Leu Asn Thr Leu Ser
195 200 205
Phe Val Cys Pro Trp Cys Ala Ser Gln Gln Thr Ser Gly His His His
210 215 220
His His His
225

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 837 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
Protein D 1/3 E6 - His HPV 18

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

ATGGATCCAA GCAGCCATTC ATCAAATATG GCGAATACCC AAATGAAATC AGACAAAATC
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ATTATTGCTC ACCGTGGTGC TAGCGGTTAT TTACCAGAGC ATACGTTAGA ATCTAAAGCA
120
CTTGCGTTTG CACAACAGGC TGATTATTTA GAGCAAGATT TAGCAATGAC TAAGGATGGT
180
CGTTTAGTGG TTATTCACGA TCACTTTTTA GATGGCTTGA CTGATGTTGC GAAAAAATTC
240
CCACATCGTC ATCGTAAAGA TGGCCGTTAC TATGTCATCG ACTTTACCTT AAAAGAAATT
300
CAAAGTTTAG AAATGACAGA AAACTTTGAA ACCATGGCGC GCTTTGAGGA TCCAACACGG
360
CGACCCTACA AGCTACCTGA TCTGTGCACG GAACTGAACA CTTCACTGCA AGACATAGAA
420
ATAACCTGTG TATATTGCAA GACAGTATTG GAACTTACAG AGGTATTTGA ATTTGCATTT
480
AAAGATTTAT TTGTGGTGTA TAGAGACAGT ATACCGCATG CTGCATGCCA TAAATGTATA
540
GATTTTTATT CTAGAATTAG AGAATTAAGA CATTATTCAG ACTCTGTGTA TGGAGACACA
600
TTGGAAAAAC TAACTAACAC TGGGTTATAC AATTTATTAA TAAGGTGCCT GCGGTGCCAG
660
AAACCGTTGA ATCCAGCAGA AAAACTTAGA CACCTTAATG AAAAACGACG ATTTCACAAC
720
ATAGCTGGGC ACTATAGAGG CCAGTGCCAT TCGTGCTGCA ACCGAGCACG ACAGGAACGA
780
CTCCAACGAC GCAGAGAAAC ACAAGTAACT AGTGGCCACC ATCACCATCA CCATTAA
837

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 279 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
Protein D 1/3 E6 - His HPV 18

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Met Asp Pro Ser Ser His Ser Ser Asn Met Ala Asn Thr Gln Met Lys
1 5 10 15
Ser Asp Lys Ile Ile Ile Ala His Arg Gly Ala Ser Gly Tyr Leu Pro
20 25 30
Glu His Thr Leu Glu Ser Lys Ala Leu Ala Phe Ala Gln Gln Ala Asp
35 40 45

Tyr Leu Glu Gln Asp Leu Ala Met Thr Lys Asp Gly Arg Leu Val Val
50 55 60
Ile His Asp His Phe Leu Asp Gly Leu Thr Asp Val Ala Lys Lys Phe
65 70 75 80
Pro His Arg His Arg Lys Asp Gly Arg Tyr Tyr Val Ile Asp Phe Thr
85 90 95
Leu Lys Glu Ile Gln Ser Leu Glu Met Thr Glu Asn Phe Glu Thr Met
100 105 110
Ala Arg Phe Glu Asp Pro Thr Arg Arg Pro Tyr Lys Leu Pro Asp Leu
115 120 125
Cys Thr Glu Leu Asn Thr Ser Leu Gln Asp Ile Glu Ile Thr Cys Val
130 135 140
Tyr Cys Lys Thr Val Leu Glu Leu Thr Glu Val Phe Glu Phe Ala Phe
145 150 155 160
Lys Asp Leu Phe Val Val Tyr Arg Asp Ser Ile Pro His Ala Ala Cys
165 170 175
His Lys Cys Ile Asp Phe Tyr Ser Arg Ile Arg Glu Leu Arg His Tyr
180 185 190
Ser Asp Ser Val Tyr Gly Asp Thr Leu Glu Lys Leu Thr Asn Thr Gly
195 200 205
Leu Tyr Asn Leu Leu Ile Arg Cys Leu Arg Cys Gln Lys Pro Leu Asn
210 215 220
Pro Ala Glu Lys Leu Arg His Leu Asn Glu Lys Arg Arg Phe His Asn
225 230 235 240
Ile Ala Gly His Tyr Arg Gly Gln Cys His Ser Cys Cys Asn Arg Ala
245 250 255
Arg Gln Glu Arg Leu Gln Arg Arg Arg Glu Thr Gln Val Thr Ser Gly
260 265 270
His His His His His His
275

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1152 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

Protein D1/3 E6 E7 His/ HPV 18
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

ATGGATCCAA GCAGCCATTC ATCAAATATG GCGAATACCC AAATGAAATC AGACAAAATC
60
ATTATTGCTC ACCGTGGTGC TAGCGGTTAT TTACCAGAGC ATACGTTAGA ATCTAAAGCA
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CTTGCGTTTG CACAACAGGC TGATTATTTA GAGCAAGATT TAGCAATGAC TAAGGATGGT
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CGTTTAGTGG TTATTCACGA TCACTTTTTA GATGGCTTGA CTGATGTTGC GAAAAAATTC
240
CCACATCGTC ATCGTAAAGA TGGCCGTTAC TATGTCATCG ACTTTACCTT AAAAGAAATT
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480
AAAGATTTAT TTGTGGTGTA TAGAGACAGT ATACCGCATG CTGCATGCCA TAAATGTATA
540
GATTTTTATT CTAGAATTAG AGAATTAAGA CATTATTCAG ACTCTGTGTA TGGAGACACA
600
TTGGAAAAAC TAACTAACAC TGGGTTATAC AATTTATTAA TAAGGTGCCT GCGGTGCCAG
660
AAACCGTTGA ATCCAGCAGA AAAACTTAGA CACCTTAATG AAAAACGACG ATTTCACAAC
720

ATAGCTGGGC ACTATAGAGG CCAGTGCCAT TCGTGCTGCA ACCGAGCACG ACAGGAACGA
780
CTCCAACGAC GCAGAGAAAC ACAAGTAATG CATGGACCTA AGGCAACATT GCAAGACATT
840
GTATTGCATT TAGAGCCCCA AAATGAAATT CCGGTTGACC TTCTATGTCA CGAGCAATTA
900
AGCGACTCAG AGGAAGAAAA CGATGAAATA GATGGAGTTA ATCATCAACA TTTACCAGCC
960
CGACGAGCCG AACCACAACG TCACACAATG TTGTGTATGT GTTGTAAGTG TGAAGCCAGA
1020
ATTGAGCTAG TAGTAGAAAG CTCAGCAGAC GACCTTCGAG CATTCCAGCA GCTGTTTCTG
1080
AACACCCTGT CCTTTGTGTG TCCGTGGTGT GCATCCCAGC AGACTAGTGG CCACCATCAC
1140
CATCACCATT AA
1152

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 384 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
Protein D1/3 E6 E7 His/ HPV 18

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Met Asp Pro Ser Ser His Ser Ser Asn Met Ala Asn Thr Gln Met Lys
1 5 10 15
Ser Asp Lys Ile Ile Ile Ala His Arg Gly Ala Ser Gly Tyr Leu Pro
20 25 30
Glu His Thr Leu Glu Ser Lys Ala Leu Ala Phe Ala Gln Gln Ala Asp
35 40 45
Tyr Leu Glu Gln Asp Leu Ala Met Thr Lys Asp Gly Arg Leu Val Val
50 55 60
Ile His Asp His Phe Leu Asp Gly Leu Thr Asp Val Ala Lys Lys Phe
65 70 75 80
Pro His Arg His Arg Lys Asp Gly Arg Tyr Tyr Val Ile Asp Phe Thr
85 90 95
Leu Lys Glu Ile Gln Ser Leu Glu Met Thr Glu Asn Phe Glu Thr Met
100 105 110
Ala Arg Phe Glu Asp Pro Thr Arg Arg Pro Tyr Lys Leu Pro Asp Leu
115 120 125
Cys Thr Glu Leu Asn Thr Ser Leu Gln Asp Ile Glu Ile Thr Cys Val
130 135 140
Tyr Cys Lys Thr Val Leu Glu Leu Thr Glu Val Phe Glu Phe Ala Phe
145 150 155 160
Lys Asp Leu Phe Val Val Tyr Arg Asp Ser Ile Pro His Ala Ala Cys
165 170 175
His Lys Cys Ile Asp Phe Tyr Ser Arg Ile Arg Glu Leu Arg His Tyr
180 185 190
Ser Asp Ser Val Tyr Gly Asp Thr Leu Glu Lys Leu Thr Asn Thr Gly
195 200 205
Leu Tyr Asn Leu Leu Ile Arg Cys Leu Arg Cys Gln Lys Pro Leu Asn
210 215 220
Pro Ala Glu Lys Leu Arg His Leu Asn Glu Lys Arg Arg Phe His Asn
225 230 235 240
Ile Ala Gly His Tyr Arg Gly Gln Cys His Ser Cys Cys Asn Arg Ala
245 250 255
Arg Gln Glu Arg Leu Gln Arg Arg Arg Glu Thr Gln Val Met His Gly
260 265 270
Pro Lys Ala Thr Leu Gln Asp Ile Val Leu His Leu Glu Pro Gln Asn
275 280 285
Glu Ile Pro Val Asp Leu Leu Cys His Glu Gln Leu Ser Asp Ser Glu
290 295 300

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Glu Glu Asn Asp Glu Ile Asp Gly Val Asn His Gln His Leu Pro Ala
305                 310                 315                 320
Arg Arg Ala Glu Pro Gln Arg His Thr Met Leu Cys Met Cys Cys Lys
                325                 330                 335
Cys Glu Ala Arg Ile Glu Leu Val Val Glu Ser Ser Ala Asp Asp Leu
            340                 345                 350
Arg Ala Phe Gln Gln Leu Phe Leu Asn Thr Leu Ser Phe Val Cys Pro
        355                 360                 365
Trp Cys Ala Ser Gln Gln Thr Ser Gly His His His His His His
    370                 375                 380
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(57) Abstract

A transcutaneous immunization system where the topical application of an adjuvant and an antigen or nucleic acid encoding for an antigen, to intact skin induces a systemic or mucosal antibody response. The immune response so elicited can be enhanced by physical or chemical skin penetration enhancement.

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USE OF SKIN PENETRATION ENHANCERS AND BARRIER DISRUPTION AGENTS TO ENHANCE THE TRANSCUTANEOUS IMMUNE RESPONSE INDUCED BY ADP-RIBOSYLATING EXOTOXIN

BACKGROUND OF THE INVENTION

1. Field of the Invention

The invention relates to transcutaneous immunization using an ADP-ribosylating exotoxin or other adjuvants with an antigen, and the use of penetration enhancers and barrier disruption agents to enhance the immune response. The invention also relates to activation of the antigen, adjuvant, their targets in the skin, or a combination thereof to enhance the antigen-specific immune response induced thereto.

2. Description of the Related Art

Skin, the largest organ of the human body, is an important part of the body's defense against invasion by infectious agents and contact with noxious chemicals (see Bos, 1997). Unwanted skin reactions such as allergic or atopic dermatitis are known, but induction of a systemic immune response by application of an adjuvant and antigen which elicits specific immune effectors and provides a therapeutic advantage by simple application of adjuvant and antigen to skin does not appear to have been taught or suggested prior to our invention.

Cholera toxin (CT) and heat labile interotoxin from E. coli (LT) are examples of a noxious chemical, which one would have expected the protective layers of skin to protect against penetration by the noxius substances. Craig (1965) reported that stool filtrates of cholera patients injected intracutaneously into rabbits or guinea pigs produced a characteristic delayed, sustained edematous induration (swelling), which was induced by the presence of toxin in the skin. The swelling and vascular leakage was so dramatic that it was ascribed to an unknown permeability factor which was later shown to be CT itself. Thus, one could have reasonably expected that CT would be extremely reactogenic when placed on the skin, if it were to enter the skin, causing similar redness and swelling. The Craig test injecting CT into the skin, became a standard measurement for the presence and amount of CT in stool filtrates or culture

media. Data confirmed that this skin reactivity was due to cholera toxin (see Finkelstein and LoSpallutto, 1969).

Craig (1965) cautioned, "The absence of skin lesions in clinical cholera certainly does not preclude the possibility that the noxa responsible for gut damage could also have a deleterious effect upon the skin provided it is applied to skin in sufficient concentration". The extreme reactogenicity of cholera toxin in the skin was used as a test for its toxicity and the prior art evidenced an expectation that cholera toxin would be reactogenic if applied to the skin, producing an undesirable reaction. Such adverse reactions have been well documented by known authorities in the field (Craig, 1972).

In contrast, we have shown cholera toxin to be immunogenic, acting as both antigen and adjuvant, when placed on the skin but without any resulting local or systemic side effects. This lack of reactogenicity when cholera toxin was placed on the skin for transcutaneous immunization was surprising and contradicted conclusions one would have drawn from the prior art. Specifically, CT placed on the skin according to our invention acts as a non-toxic, non-reactogenic adjuvant, in contrast to the expectations of Craig, while injection of CT into the skin results in swelling and redness. Thus, it was not obvious prior to our invention that cholera toxin or other ADP-ribosylating exotoxins or allow adjuvants applied topically would be useful for transcutaneous immunization. In fact large doses of heat labile enterotoxin (LT) placed on the skin of humans has been shown to induce a systemic immune response without local or systemic toxicity.

This unexpected absence of reactogenicity is extremely important to the use of vaccines. Vaccine antigens and adjuvants are useful when imunization produces a protective immune response without significant unwanted reactions. Historically, reactogenicity of vaccines such as swelling, tenderness and pain at the site of injection has in some cases (e.g., typhoid and pertussis) been accepted because of the benefits of vaccination. However, high levels of reactogenicity and other side effects are not desirable, and would be problematic for development of new vaccine adjuvant and antigen candidates. Research efforts are focused on making vaccine adjuvants that are stimulatory and do not inducing unwanted reactions. Whole cell pertussis vaccines induce systemic and local side effects and, as a result, this effective vaccine and time

tested vaccine is being replaced by acellular pertussis vaccines solely because they are less reactogenic.

The present invention differs from that of Patent No. 5,830,877 which teaches the use of a naked plasmid that encodes for biologically active peptides into a mammalian host. The invention described herein teaches the use of an adjuvant and antigen or nucleic acid administered together on the skin to induce an immune response. The invention described herein further differs from that of Patent # 5,830,877 which teaches away from the use of peptides that are not encoded in a nucleic acid and produced by the host cell because of the toxicity associated with biologically active peptides, the problems and cost of isolating, purifying and synthesizing peptides and their short half life in vivo resulting form degradation by proteases present in the target tissue. This clearly teaches away from the addition of an adjuvant such a cholera toxin to a coadministered antigen or nucleic acid. In fact the novelty of the ability of a large molecule such as CT to induce an immune response by application through the skin without toxicity has led to a number of scientific papers describing this novelty and public excitement over the potential implication of delivery of proteins for vaccination by skin application. Unlike Patent No. 5,830,877 the present invention is not dependent on stimulation of local inflammation or irritation. Unlike Patent No. 5,830,877 the invention does not depend on irritation or local inflammation to increase the permeability of cell membranes to enhance the uptake of the antigens, plasmids or RNA. In fact the striking feature regarding Transcutaneous Immunization is the absence of local inflammation.

Unlike the present invention, Patent No. 5,824,313 teaches the application of extremely small (less than 500 daltons) lymphoid organ modifying agents such as 1,25-dihydroxy-16-ene Vitamin D.sub.3 and calcipotriene or dehydroepiandrosterone (DHEA), DHEA congeners and DHEA-derivatives with the intramuscular injection of an antigen to affect antibody responses.

Transcutaneous immunization requires both passage of an antigen through the outer barriers of the skin, which was thought to be impervious to such passage, and an immune response to the antigen. Fisher's Contact Dermatitis states that molecules of greater than 500 daltons cannot normally penetrate the skin. There is a report by Paul et al. (1995) of induction of an immune response with transferosomes, a lipid structure

distinct from liposomes. In this publication, the transferosomes were used as a vehicle for antigen (bovine serum albumin and gap junction proteins) and complement-mediated lysis of antigen-sensitized liposomes was assayed. The limit to penetration of the skin by antigen was stated to be 750 daltons. In their study, an immune response was not induced when a solution containing antigen was placed on the skin; only transferosomes were able to induce an immune response. Paul and Cvec (1995) also stated that it is "impossible to immunize epicutaneously with simple peptide or protein solutions".

Such references explain why our successful use of a molecule like cholera toxin (which is 85,000 daltons) as an antigen or adjuvant in immunization was greeted with surprise by the field because such large molecules were not expected to penetrate the skin and, therefore, would not be expected to induce a specific immune response.

However, we have shown in U.S. Appln. No. 08/749,164 (filed November 14, 1996); U.S. Appln. No. 08/896,085 (filed July 17, 1997); and international application PCT/US97/21324 (filed November 14, 1997) that using an ADP-ribosylating exotoxin, such as cholera toxin, as an antigen could elicit a strong antibody response that is highly reproducible. When an ADP-ribosylating exotoxin, such as cholera toxin, was used as an immunoadjuvant and applied to the skin in a saline solution with a separate antigen (e.g., diphtheria toxoid), a systemic and mucosal antigen-specific antibody response could be elicited. In the present application, we disclose that transcutaneous immunization using a penetration enhancer, a barrier disruption agent, or combinations thereof may improve the adjuvant activity of a bacterial exotoxin.

We have shown that like cholera toxin (CT), heat-labile enterotoxin from E. coli (LT), Pseudomonas exotoxin A (ETA), pertussis toxin (PT) and a wide variety of antigens including killed rabies virus, recombinants such as HIV p55 gag, polysaccheride conjugates such as Hib, sonicates, pertactin for example, are able to pass through the skin and induce an immune response. Additionally CT, LT, ETA and PT and bacterial DNA and cytobines, can act as adjuvants to induce an immune response to antigens co-administered on the skin. Thus tetanus toxoid, not immunogenic by itself on the skin, can induce a strong immune response when placed on the skin with CT. We have proposed that the Langerhans cell population underlying the site of application are a preferred antigen presenting cell for delivering

antigen to the immune system. Adjuvant may act on the antigen presenting cell directly, or through lymphocytes recognizing antigen.

We propose to enhance the immune response to transcutaneously adjuvant and/or antigen utilizing penetration enhancement techniques. According to Hurley, "Skin owes its durability to the dermis, but its chemical impermeability resides in the epidermis and almost exclusively in its dead outer layer, the stratum corneum". For transcutaneous immunization using, for example, an ADP-ribosylating exotoxin as adjuvant and a soluble protein antigen such as diphtheria toxoid, penetration of the stratum corneum must occur. Penetration enhancement techniques would be designed to increase the movement of transcutaneous antigens and adjuvants through the stratum corneum layer of skin.

Further, we propose that transcutaneous immunization using activation of at least one of the antigen, adjuvant and skin component will enhance the immune response as assayed by quantitative and qualitative parameters. Antigen-adjuvant of the formulation may be activated by trypsin cleavage of a bacterial exotoxin (e.g., trypsin-cleaved LT, with or without reduction). Activation of the skin at the application site of the formulation may be accomplished by the use of barrier disruption agents (e.g., acetone, alcohol) which increases the size or activation of the underlying Langerhans cell population, or by an enzyme or combination of enzymes (e.g., enzymes with sialidase activity) which increases the amount or accessibility of ganglioside GM1 receptor.

SUMMARY OF THE INVENTION

An object of the invention is to provide an enhanced system for transcutaneous immunization which induces an immune response (e.g., humoral and/or cellular effector) in a subject, a subject being an animal or human. This delivery system provides simple application to intact skin of an organism of a formulation comprised of antigen and adjuvant to induce a specific immune response against the antigen. Although not required for induction of an immune response by this simple delivery system, supplementation of the aforementioned process with penetration enhancement or barrier disruption may enhance immunization and/or vaccination.

In particular, the adjuvant or antigen or skin may assist in the penetration of the stratum corneum or epidermis to encounter the antigen presenting cells of the immune system (e.g., Langerhans cells in the epidermis, dermal dendritic cells, dendritic cells, follicular dendritic cells, macrophages, B lymphocytes) and/or induce the antigen presenting cells to phagocytose the antigen. The antigen presenting cells then present the antigen to T and B cells. In the instance of Langerhans cells, the antigen presenting cells then may migrate from the skin to the lymph nodes and present antigen to lymphocytes (e.g., B and/or T cells), thereby inducing an antigen-specific immune response.

In addition activation of the antigen, adjuvant, skin, or any combination thereof may be accomplished to supplement the immunization process.

In addition to eliciting immune reactions leading to generation of an antigen-specific B lymphocyte and/or T lymphocyte, including a cytotoxic T lymphocyte (CTL), another object of the invention is to positively and/or negatively regulate components of the immune system by using the transcutaneous immunization system to affect antigen-specific helper (Th1 and/or Th2) or delayed-type hypersensitivity (DTH) T-cell subsets. This can be exemplified by the differential behavior of CT and LT which can result in different T-helper responses or different levels of protection in in-vivo challenge molds using transcutaneous immunization.

DESCRIPTION OF THE DRAWINGS

Figures 1a-f are photographs showing no inflammation at the site of immunization (A,B), Langerhans cell activation by LT in human skin at the site of immunization (C,E) and the absence of Langerhans cell activation in skin from the contralateral arm (D,F).

Figures 2a-d are photographs showing normal Langerhans cell (A,B, 200 and 400X) and Langerhan cell activation by Cholera Toxin in mouse skin (C,D. 200X, 400X).

DESCRIPTION OF PREFERRED EMBODIMENTS

In one embodiment of the invention, a formulation containing antigen and adjuvant such as CT and DT, is applied to intact skin of an organism after penetration enhancement of the skin, the antigen is presented to immune cells, and an antigen-specific immune response is induced without perforating the skin. The formulation may include additional antigens or nucleic acids such that transcutaneous application of the formulation induces an immune response to multiple antigens, or nucleic acids encoding for antigens preferably from 2 to 20 but possibly up to 200. In such a case, the antigens may or may not be derived from the same source, but the antigens will have different chemical structures so as to induce immune responses specific for the different antigens. Antigen-specific lymphocytes may participate in the immune response and, in the case of participation by B lymphocytes, antigen-specific antibodies may be part of the immune response.

In another embodiment of the invention, the invention is used to treat an organism. If the antigen is derived from a pathogen, the treatment vaccinates the organism against infection by the pathogen or against its pathogenic effects such as those caused by toxin secretion. A formulation that includes a tumor antigen may provide a cancer treatment; a formulation that includes an allergen may be used to treat for allergic disease; a formulation that includes an autoantigen may provide a treatment for a disease caused by the organism's own immune system (e.g., autoimmune disease). The invention may be used therapeutically to treat existing disease, protectively to prevent disease, or to reduce the severity and/or duration of disease.

In a further embodiment of the invention, a patch for use in the above methods is provided. The patch may comprise a dressing, and effective amounts of antigen or nucleic acids and adjuvant. The dressing may be occlusive or non-occlusive. The patch may contain penetration enhancers or may include a device for physical penetration enhancement. The patch may include additional antigens such that application of the patch induces an immune response to multiple antigens. In such a case, the antigens may or may not be derived from the same source, but the antigens will have different chemical structures so as to induce an immune response specific

for the different antigens. For effective treatment, multiple patches may be applied at frequent intervals or constantly over a period of time.

Moreover, in yet another embodiment of the invention, the formulation is applied to intact skin overlying more than one draining lymph node field using either single or multiple applications or a separate patch for adjuvant or antigen/nucleic acid. The formulation may include additional antigens such that application to intact skin induces an immune response to multiple antigens. In such a case, the antigens may or may not be derived from the same source, but the antigens will have different chemical structures so as to induce an immune response specific for the different antigens.

The formulation may be applied to the skin to boost or prime the immune response in conjunction with other routes of immunization. Thus, priming with transcutaneous immunization with either single or multiple applications may be followed with oral, nasal, or parenteral techniques for boosting immunization with the same or altered antigens. The formulation may include additional antigens such that application to intact skin induces an immune response to multiple antigens. In such a case, the antigens may or may not be derived from the same source, but the antigens will have different chemical structures so as to induce an immune response specific for the different antigens.

In addition to antigen and activated adjuvant, the formulation may comprise a vehicle. For example, the formulation may comprise AQUAPHOR (an emulsion of petrolatum, mineral oil, mineral wax, wool wax, panthenol, bisabol, and glycerin), emulsions (e.g., aqueous creams), microemulsions, gels, oil-in-water emulsions (e.g., oily creams), anhydrous lipids and oil-in-water emulsions, anhydrous lipids and water-in-oil emulsions, fats, waxes, oil, silicones, and humectants (e.g., glycerol).

The antigen may be derived from a pathogen that can infect the organism (e.g., bacterium, virus, fungus, or parasite), or a cell (e.g., tumor cell or normal cell) or allergen or biological warfare agent. The antigen may be a tumor antigen or an autoantigen. Chemically, the antigen may be a carbohydrate, glycolipid, glycoprotein, lipid, lipoprotein, phospholipid, polypeptide, or fusion protein (recombinant) or chemical conjugate of the above. The molecular weight of the antigen may be greater than 500 daltons, preferably greater than 800 daltons, and more preferably greater than 1000 daltons.

Antigen may be obtained by recombinant means, chemical synthesis, or purification from a natural source. One advantage of transcutaneous immunization may be that purification of an antigen is not necessary e.g. a whole organism may be sonicated and used for immunization. The level of toxicity associated with injecting a product from such a preparation is often too toxic to be tolerated, such as LPS, which can be fatal if injected, but is non-toxic on the skin. Preferred are proteinaceous antigen or conjugates with polysaccharide. Antigen may be at least partially purified in cell-free form. Alternatively, antigen may be provided in the form of a live virus, an attenuated live virus, or an inactivated virus, sonicated or lysed whole bacterium, parasite or detergent treated virus, or fraction thereof.

Inclusion of an adjuvant may allow potentiation or modulation of the immune response. Moreover, selection of a suitable antigen or adjuvant may allow preferential induction of a humoral or cellular immune pr mucosal response, specific antibody isotypes (e.g., IgM, IgD, IgA1, IgA2, IgE, IgG1, IgG2, IgG3, and/or IgG4), and/or specific T-cell subsets (e.g., CTL, Th1, Th2 and/or T_{DTH}). Optionally, antigen, adjuvant, may be provided in the formulation by means of a nucleic acid (e.g., DNA, RNA, cDNA, cRNA) encoding the antigen or adjuvant as appropriate with a antigen or adjuvant that has been added to the nucleic acid. This technique is called genetic immunization.

The term "antigen" as used in the invention, is meant to describe a substance that induces a specific immune response when presented to immune cells of an organism. An antigen may comprise a single immunogenic epitope, or a multiplicity of immunogenic epitopes recognized by a B-cell receptor (i.e., antibody on the membrane of the B cell) or a T-cell receptor. A molecule may be both an antigen and an adjuvant (e.g., cholera toxin) and, thus, the formulation may contain only one component. Antigen may be provided as a whole organism such as, for example, a bacterium or virion; antigen may be obtained from an extract or lysate, either from whole cells or membrane alone; or antigen may be chemically synthesized for produced by recombinant means.

The term "adjuvant" as used in the invention, is meant to describe a substance added to the formulation to assist in inducing an immune response to the antigen.

The term "effective amount" as used in the invention, is meant to describe that amount of antigen which induces an antigen-specific immune response. Such

induction of an immune response may provide a treatment such as, for example, immunoprotection, desensitization, immunosuppression, modulation of autoimmune disease, potentiation of cancer immunosurveillance, or therapeutic vaccination against an established infectious disease.

By the epidermis we mean the cells of the skin from the basal layer of kertinoctyes and basal lamina up to and through the stratum corneum.

The definition of transdermal is generally held to be: Relating to, being, or supplying a medication in a form for absorption through the skin into the bloodstream (~drug delivery) (~nitroglycerin) (~nicotine patch). [2]Frederick C. Mish et al., eds., *Merriam-Webster's Collegiate Dictionary,* 10th ed. (Springfield, MA.: Merriam-Webster, Incorporated, 1997), 861.

The term draining lymph node field as used in the invention means an anatomic area over which the lymph collected is filtered through a set of defined set of lymph nodes (e.g., cervical, axillary, inguinal, epitrochelear, popliteal, those of the abdomen and thorax).

Skin penetration may be enhanced using techniques that increase skin hydration. According to Roberts and Walker (1993), "The state of hydration of the stratum corneum (SC) is one of the most important factors in determining the rate of percutaneous absorption of a given solute". The state of hydration interacts with the principal of diffusion in determining the rate of absorption of a substance through the skin. Furthermore, Hurley stated:

> "Absorption of substances through the stratum corneum is believed to occur by diffusion in accordance with Fick's laws of diffusion in which the rate of absorption of a chemical is proportional to the concentration difference across the membrane. Thus, a concentration gradient between the high concentration of solute on the skin surface and its absence or low concentration below the stratum corneum is the driving force in this process. Transcorneal movement of absorption is classically represented as 'percellular', that is, directly through the cell walls of the compacted corneum and not intercellularly. Intracellular protein filaments are described as the pathways for polar (water soluble) compounds and the medium between filaments serves as the route for nonpolar (lipid-soluble) substances. . . Hydration increases the permeability of the stratum corneum for most substances by a number of cytophysical mechanisms that are not completely clarified."

Thus, while skin hydration is generally known to enhance skin penetration, the mechanisms by which this occurs are not entirely clear and, thus, not predictable prior

to the present invention and not thought to allow penetration of large molecules (7750 daltons).

The use of vehicles for increasing hydration is well known. Occlusive dressings, such as vapor-impenetrable plastic films (e.g., polyvinylidine, polyethylene) enhance absorbtion principally through increased hydration of the stratum corneum, a result of swelling of the corneocytes, and uptake of water into the intercellular corridors. Hydrocolloid patches may also be used to enhance skin penetration. Absorbtion of steroids can be increase over 100 fold using plastic occlusive film. Generally, greases, oils or impermeable plastic induce the most hydration by occlusion. See, for example, Idson (1978); Hollingsbee (1995); and Mckenzie and Stoughton (1962). The use of hydration or vehicle for hydration with an antigen and adjuvant were not known prior to our invention as penetration. The skin was thought to be limited to small molecules even in the hydrated state.

Suitable agents which are known to enhance absorption of drugs through skin are described in Sloan, Use of Solubility Parameters from Regular Solution Theory to Describe Partitioning-Driven Processes, Ch. 5, "Prodrugs: Topical and Ocular Drug Delivery" (Marcel Dekker, 1992), and at places elsewhere in the text.

It is expected that these techniques (and others which are conventionally used to facilitate drug delivery) may be adapted to preparation of nucleic acids for use in the methods of the invention by those of ordinary skill in the art without undue experimentation. Specific examples illustrating this suitability are set forth below.

The state of the art in skin penetration enhancement is described in Pharmaceutical Skin Penetration Enhancement Edited by Kenneth A. Walters and Jonathan Hadgraft, Published by Marcel Dekker, Inc., New York, 1993.

Skin permeability and/or skin hydration may be expected by selecting an appropriate vehicle from different classes, such as humectant (e.g., glycols, glycerols), powder, (e.g., clays, shake lotions), oil/water (O/W) emulsion (e.g., aqueous creams), water/oil emulsion (e.g., oily creams), emulsifying base (e.g., anhydrous lipid and O/W emulsifiers), absorbtion base (e.g., anhydrous lipid and W/O emulsifiers), lipophilic (e.g., fats, waxes, oils, silicones), and occlusive dressing (e.g., plastic wrap).

Other methods that disrupt the stratum corneum proteins to enhance penetration in the present invention may be employed. Salicylic acid is a keratinolytic that may increase absorption. Urea acts both as a keratinolytic and hydrater of the

skin, and may act as a penetration enhancer. Phospholipase A2 and phosphatidylcholine dependent phospholipase C may be used as epidermal enzymes to enhance penetration. Other penetration enhancers may include ethanol, acetone, detergents, bases, nair©, propylene glycol, pyrriolidones, dimethylacetamide, dimethylformamide, dimethylsulfoxide, alkyl sulfoxide, phosphine oxide, surfactants and caprolactams such as azone. Other compounds that may be used for penetration enhancement include amines and amides, alkyl N,N-distributed-amino acetates, decylmethylsulfoxide, pyrrolidones, pirotiodecane (HPE-101), benzlyalkonium, benzylalkonium chloride polymers, silicone based polymers, fatty acids, cyclic ureas, terpenes, liposomes, and cyclodextrins. Penetration enhancers are well known in the art, for example as described in Pharmaceutical Penetration Enhancement, (Marcel Dekker, 1993) Other techniques that may be employed for penetration include iontophoresis, ultrasound, electroporation, tape stripping, the use of gene guns or other propellant devices, tines such as used for TB tine tests (as provided by Mono-Vacc system) or microneedles which penetrate the outer surface of the skin, or abrasives which remove the outer layers of the skin and lipid extraction.

A device which may be used for disruption of the stratum corneum (which is distributed in the U.S. by Connaught Laboratories, Inc. of Swiftwater, Pa.) consists of a plastic container having a syringe plunger at one end and a tyne disk at the other. The tyne disk supports a multiplicity of narrow diameter tynes of a length which will just scratch the outermost layer of epidermal cells but not penetrate the epidermis. Each of the tynes in the MONO-VACC kit is coated with old tuberculin; in the present invention, each needle may be coated with a pharmaceutical composition of antigen/nucleic acid and adjuvant. Use of the device with the present invention may not be according to the manufacturer's written instructions included with the device product because when used with the present invention does not penetrate the epidermis. Hereto, the device may be used for surface disruption to disrupt the outermost layes of the skin, the stratum corneum and upper epidermis, to enhance the transcutaneous immunization. Similar devices which may also be used in this embodiment are those which are currently used to perform allergy tests.

Other approaches include barrier disruption. Inhibition of cholesterol synthesis using systemically administered HMG CoA reductase inhibitors and similar

drugs interfere with barrier function and may allow enhanced penetration of the formulation component.

It is also conceivable that the skin can be transformed to enhance the transcutaneous immune response. CT and LT exert their effect via the ganglioside GM1 binding by the B subunit. Ganglioside GM1 is a ubiquitous cell membrane glycolipid found in all mammalian cells. In the gastrointestinal tract, when the pentameric CT B subunit binds to the cell surface, a hydrophilic pore is formed which allows the A subunit to penetrate across the lipid bilayer. The skin contains gangliosides at a concentration of 30-35 nmol NeuAC/gm. Skin gangliosides are possible targets for initiating transcutaneous immunization via mechanisms such as Langerhans cells activation as described above.

One possible method to activate the skin for enhancing the effect of transcutaneous immunization with ADP ribosylating exotoxins by increasing the number of GM1 ganglioside molecules in skin cells. This could be achieved by activation of receptor cells using sialidase to convert gangliosides that do not bind the toxin into the sialidase-stable cholera toxin binding ganglioside GGnSLC (ganglioside GM1):

> "It is interesting that the cholera vibrio is perhaps the best-known source of sialidase (or neuraminidase, as it is often called). Could this sialidase play a part in the natural history of the disease by making more receptors available for the toxin? If so, should any active immunizing agent the disease contain an anti-neuriminidase element? Incubation of intestinal scrapings with sialidase leads to a considerable increase in their ability to bind the toxin, which is due not only to conversion of sialidase-labile ganglioside to cholera toxin-binding ganglioside, but also, apparently to the unmasking of otherwise unapproachable ganglioside binding sites possibly by breaking down glycoproteins. Pretreatment of dog intestine with sialidase makes it produce more fluid in response to cholera toxin; treatment of adrenal cells with sialidase increases their responsiveness to cholera toxin; pretreatment of pigeon red cells with sialidase increases the activation of the adenylate cyclase in them by cholera toxin.

The biochemistry of cholera, In: Cholera: The American Scientific Experience, 1947-1980, van Heyningen, W.E., and Seal, J.R., Eds, Waterview Press, Boulder, 1983, page 263 (citations omitted).

The effect of treatment of the skin with sialidase may enhance the binding of an ADP ribosylating exotixin such as CT to the immune cells targeted by transcutaneous immunization. This represents a kind of activation of the skin for transcutaneous immunization. Additionally, neuraminidase may act as an epidermal enzyme concurrently enhancing penetration.

The use of a penetration enhancer may be used in conjunction with activation of the skin. Activation of the skin for transcutaneous immunization may also be after treatments such as acetone or alcohol swabbing. It has been shown that skin barrier disruption using acetone swabbing of the skin increased Langerhans cell density by 80% and increased the reaction to contact allergens in vivo. If the density of Langerhans cells is increased, then the potency of the immune response may be increased. Similar chemical disruption might be expected to increase the number of Langerhans cells and result in activation of the skin component of transcutaneous immunization, by tape stripping, sodium dodecyl sulfate, the use of alcohol swabbing, or a depiliatory such as calcium hydroxide. See Proksch and Brasch (1996, 1997) for use of penetration enhancers and barrier disruption in allergic contact dermatitis.

Penetration enhancement may be achieved by performance of simple maneuvers such as alcohol swabbing immediately prior to immunization, by concurrent use of penetration enhancement compounds or techniques or by techniques such as acetone swabbing 24 hours prior to increase the number of Langerhans cells.

Processes for preparing a pharmaceutical formulation are well-known in the art, whereby the antigen and adjuvant is combined with a pharmaceutically acceptable carrier vehicle. Suitable vehicles and their preparation are described, for example, in Remington's Pharmaceutical Sciences by E.W. Martin. Such formulations will contain an effective amount of the antigen and adjuvant together with a suitable amount of vehicle in order to prepare pharmaceutically acceptable compositions suitable for administration to a human or animal. The formulation may be applied in the form of an cream, emulsion, gel, lotion, ointment, paste, solution, suspension, or other forms known in the art. In particular, formulations that enhance skin hydration, penetration, or both are preferred. There may also be incorporated other pharmaceutically acceptable additives including, for example, diluents, excipients, binders, stabilizers, preservatives, and colorings.

Without being bound to any particular theory but only to provide an explanation for our observations, it is presumed that the transcutaneous immunization delivery system carries antigen to cells of the immune system where an immune response is induced. The antigen may pass through the normal protective outer layers of the skin (i.e., stratum corneum) and induce the immune response directly, or through an antigen presenting cell (e.g., macrophage, tissue macrophage, Langerhans cell, dendritic cell, dermal dendritic cell, B lymphocyte, or Kupffer cell) that presents processed antigen to a T lymphocyte (see Stingl et al., 1989; Streilein and Grammer, 1989; Tew et al., 1997). Optionally, the antigen may pass through the stratum corneum via a hair follicle or a skin organelle (e.g., sweat gland, oil gland).

Transcutaneous immunization with bacterial ADP-ribosylating exotoxins (bAREs) may target the epidermal Langerhans cell, known to be among the most efficient of the antigen presenting cells (APCs). We have found that bAREs activate Langerhans cells when applied epicutaneously to the skin in saline solution. Adjuvants such as activated LT may greatly enhance Langerhans cell activation. The Langerhans cells direct specific immune responses through phagocytosis of the antigens, and migration to the lymph nodes where they act as APCs to present the antigen to lymphocytes, and thereby induce a potent antibody response. Although the skin is generally considered a barrier to invading organisms, the imperfection of this barrier is attested to by the numerous Langerhans cells distributed throughout the epidermis that are designed to orchestrate the immune response against organisms invading via the skin. According to Udey (1997):

> "Langerhans cells are bone-marrow derived cells that are present in all mammalian stratified squamous epithelia. They comprise all of the accessory cell activity that is present in uninflammed epidermis, an in the current paradigm are essential for the initiation and propagation of immune responses directed against epicutaneously applied antigens. Langerhans cells are members of a family of potent accessory cells ('dendritic cells') that are widely distributed, but infrequently represented, in epithelia and solid organs as well as in lymphoid tissue.
>
> "It is now recognized that Langerhans cells (and presumably other dendritic cells) have a life cycle with at least two distinct stages. Langerhans cells that are located in epidermis constitute a regular network of antigen-trapping 'sentinel' cells. Epidermal Langerhans cells can ingest particulates, including microorganisms, and are efficient processors of complex antigens. However, they express only low levels of MHC class I and II antigens and costimulatory molecules

(ICAM-1, B7-1 and B7-2) and are poor stimulators of unprimed T cells. After contact with antigen, some Langerhans cells become activated, exit the epidermis and migrate to T-cell-dependent regions of regional lymph nodes where they local as mature dendritic cells. In the course of exiting the epidermis and migrating to lymph nodes, antigen-bearing epidermal Langerhans cells (now the 'messengers') exhibit dramatic changes in morphology, surface phenotype and function. In contrast to epidermal Langerhans cells, lymphoid dendritic cells are essentially non-phagocytic and process protein antigens inefficiently, but express high levels of MHC class I and class II antigens and various costimulatory molecules and are the most potent stimulators of naive T cells that have been identified."

We envision that the potent antigen presenting capability of the epidermal Langerhans cells can be exploited for transcutaneously delivered vaccines. A transcutaneous immune response using the skin immune system would require delivery of vaccine antigen only to Langerhans cells in the stratum corneum (the outermost layer of the skin consisting of cornified cells and lipids) via passive diffusion and subsequent activation of the Langerhans cells to take up antigen, migrate to B-cell follicles and/or T-cell dependent regions, and present the antigen to B and/or T cells. If antigens other that bAREs (for example diptheria toxoid) are to be phagocytosed by the Langerhans cells, then these antigens could also be taken to the lymph node for presentation to T-cells and subsequently induce an immune response specific for that antigen (e.g., diptheria toxoid). Thus, a feature of transcutaneous immunization is the activation of the Langerhans cell, presumably by bacterial ADP-ribosylating exotoxins, ADP-ribosylating exotoxin binding subunits (e.g., cholera toxin B subunit), or other adjuvants or Langerhans cell activating substance. Increasing the skin population of Langerhans cells using strategies such as acetone swabbing could then be expected to enhance the transcutaneous immune response.

The spectrum of more commonly known skin immune responses is represented by contact dermatitis and atopy. Contact dermatitis, a pathogenic manifestation of LC activation, is directed by Langerhans cells which phagocytose antigen, migrate to lymph nodes, present antigen, and sensitize T cells that migrate to the skin and cause the intense destructive cellular response that occurs at affected skin sites (Dahl, 1996; Leung, 1997). Atopic dermatitis may utilize the Langerhans cell in a similar fashion, but is identified with Th2 cells and is generally associated with high levels of IgE antibody (Dahl, 1996; Leung, 1997).

Transcutaneous immunization with cholera toxin and related bAREs on the other hand is a novel immune response with an absence of superficial and microscopic post-immunization skin findings (i.e., non-inflamed skin) shown by the absence of lymphocyte infiltration 24, 48 and 120 hours after immunization. This is strikingly shown by completion of a Phase I trial in which humans were immunized with LT under a simple occlusive patch. Potent anti-LT IgG and IgA antibodies were stimulated. Two volunteers had biopsies performed at the site of immunization. Micro-scopic evaluation confirmed the clinical observation that no inflammation was seen. This suggests that Langerhans cells, which "comprise all of the accessory cell activity that is present in uninflammed epidermis, and in the current paradigm are essential for the initiation and propagation of immune responses directed against epicutaneously applied antigens" (Udey, 1997) may have been recruited. The uniqueness of the transcutaneous immune response here is also indicated by the both high levels of antigen-specific IgG antibody, and the type of antibody produced (e.g., IgG1, IgG2a, IgG2b, IgG3 and IgA) and the absence of anti-CT IgE antibody. However, other immune cells may be engaged and speculation on the mechanism should not limit the invention.

Thus, we have found that bacterial-derived toxins applied to the surface of the skin can activate Langerhans cells and that TCI induces a potent immune response manifested as high levels of antigen-specific circulating IgG antibodies and would expect that penetration enhancement would enhance the immune response. Transcutaneous adjuvant and penetration enhancer may be used in transcutaneous immunization to enhance the IgG antibody or T-cell response to proteins not otherwise immunogenic by themselves when placed on the skin.

Transcutaneous targeting of Langerhans cells may also be used to deactivate their antigen presenting function, thereby preventing immunization or sensitization. Techniques to mobilize Langerhans cells or other skin immune cells yet negatively modulate them include, for example, the use of anti-inflammatory steroidal or non-steroidal agents (NSAID), cyclophosphamide or other immunosuppressants, interleukin-10, TGFβ monoclonal antibody to interleukin-1, ICE inhibitors or depletion via superantigens such as through staphylococcal enterotoxin-A (SEA) induced epidermal Langerhans cell depletion.

Transcutaneous immunization may be induced via the ganglioside GM1 binding activity of CT, LT or subunits such as CTB. Ganglioside GM1 is a ubiquitous cell membrane glycolipid found in all mammalian cells. When the pentameric CT B subunit binds to the cell surface a hydrophilic pore is formed which allows the A subunit to penetrate across the lipid bilayer.

We have shown that transcutaneous immunization by CT or CTB may require ganglioside GM1 binding activity. When mice are transcutaneously immunized with CT, CTA and CTB, only CT and CTB resulted in an immune response. CTA contains the ADP-ribosylating exotoxin activity but only CT and CTB containing the binding activity are able to induce an immune response indicating that the B subunit was necessary and sufficient to immunize through the skin. We conclude that the Langerhans cell or other immune cells may be activated by CTB binding to its cell surface, but more activated by the concurrent presence of the A-subunit.

In addition to activation of the skin component in the immunization process of the present invention, the antigen and/or adjuvant may be activated to enhance immunization. When CT is secreted, cleavage occurs at the trypsin recognition site and the toxin is activated. LT however, is secreted with its trypsin recognition site intact. When LT is secreted in the gastrointestinal tract and thereby exposed to gastrointestinal agents such as trypsin, the proteolytically sensitive residues that join A1 and A2 subunits of LT are cleaved, allowing the A1 subunit to ADP-ribosylate G proteins and therefore exert its toxic effects. The lack of trypsin or related agents in the skin may prevent trypsin cleavage of the proteolytically sensitive residues that join A1 and A2 subunits of LT, diminishing its adjuvant activity.

These two bacterial enterotoxins have many features in common. LT and CT have the same subunit number (A2:B5) and arrangement, and the same biological mechanism of action. An amino acid sequence similarity of 75-77% is found for both chains when comparing LT and CT, and the most significant difference occurs in the respective A chains at positions 192-195. At this site the cleavage of the A chain by trypsin occurs and the site is situated between two cysteine residues that form the internal disulfide bond of the A chain. See, for example, Mekalanos et al. (1979), Spangler (1992), and Sniderman (1995). We propose that these structural differences between the molecules are a significant influence not only on their enterotoxic properties, but also on their ability to function as adjuvants.

Unlike CT produced by V. cholerae, LT is not fully biologically active when first isolated from the bacterial cell. Consistent with the A-B model for bacterial toxins, LT requires trypsin proteolysis and disulfide reduction to be fully active (Sniderman, 1995). In the absence of proteolytic processing, the enzymatically active A1 moiety is unable to dissociate from the A2 component and cannot reach its target substrate (adenylate cyclase) on the basolateral surface of the intestinal epithelial cell. This difference in activation of the isolated material results in differences in response thresholds for LT and CT in biologic systems. For instance, CT induces detectable net fluid secretion in the mouse intestine at a dose of 5 to 10 μg. LT induces detectable net secretion in this assay at 50 to 100 μg. In the rabbit ligated illeal loop, the difference is more dramatic and clear cut. Significantly however, when LT is exposed to proteolytic enzymes with trypsin-like specificity, the molecule becomes indistinguishable from CT in any biologic assay system (Clements and Finkelstein, 1979; Dickenson and Clements, 1995).

According to Spangler (1992, citations omitted):

> "Subunit A is synthesized as a single polypeptide in both V. cholerae and E. coli. CTA is proteolytically "nicked" between residues 192 and 195 during secretion from the vibrio by V. cholerae hemagglutinin/protease, giving rise to two polypeptides, A1 (Mr = 28,826) and A2 (Mr = 5,407), covalently linked through a disulfide bridge between residues 187 and 199. In contrast, LT remains in the E. coli periplasm and is not nicked. Introduced into a genetically engineered strain of V. cholerae, LT remained unnicked, although it was secreted in the same manner as CT. Proteolytic processing is therefore not a prerequisite for secretion. Purified LTh can, however, be nicked in vitro, suggesting that the mutant vibrio used by Hirst et al. contained insufficient soluble hemagglutinin to catalyze nicking, rather than indicating an inability of LTA to be nicked. CT, when introduced via an engineered plasmid into E coli, remains unnicked and cell associated in E. coli. Therefore, the defect in processing of CT and LT in E. coli is related to the failure of E. coli to nick and secrete either toxin. This defect may explain the reduced severity of E. coli-induced enteric disease when compared with cholera. In both CT and LT, the disulphide bond linking A1 to A2 remains unreduced and the toxin is therefore essentially inactive, until it enters a cell.
>
> "Both the intact A subunit and the holotoxin are relatively inactive ADP-ribosyltransferases compared with the A1 polypeptide. Catalytic activity requires the reduction of the disulfide bond (A1:Cys-187-A2:Cys-199) linking A1 to A2. The cleavage (nicking) between residues A1-Arg 192 and the start of the A2 polypeptide at A2:Met-195 takes place during secretion of CT from the vibrio. tryptic

digestion serves the purpose in vitro for LT. Reduction, which releases CTA1 from CTA2, may be accomplished by a variety of agents, usually dithiothreitol or 2-mercaptoethanol invitro or a thiol:protein oxireductase. The endogenous reducing agent and mechanisms of reduction are not known. An observed time lag of about 16 min between the apparent binding of the toxin to the membrane receptor and the first apearance of the modified substrate intracellularly may be related to the time required for this step to occur following or during insertion or translocation".

LTh stands for LT holoezyme. Thus, if trypsin-treated LT were to be used for transcutaneous immunization, we propose similar mechanisms for disrupting disulphide bonds would occur. This may be shown for trypsin activation of LT in which trypsin-activated LT is similarly potent or of greater potentcy compared to CT and much greater in potency to untreated LT in the mouse Y-1 bioassay (see Dickinson and Clements, 1995).

We propose to activate components of the formulation such as LT using trypsin or similar compounds prior to application the skin to enhance the adjuvant activity and immunogenicity of LT. Activation of LT could also be expected to enhance the immune response to LT as an antigen. The activated adjuvant for transcutaneous immunization is preferably an ADP-ribosylating exotoxin. Optionally, hydration or occlusive dressings may be used in the transcutaneous delivery system in addition to the activation of the adjuvant.

In addition, LT has an unusual affinity for carbohydrate-containing matrices. Specifically, LT binds to an array of biological molecules containing galactose, including glycoproteins and lipopolysaccharides. This lectin-like binding property of LT results in a broader receptor distribution on mammalian cells for LT than for CT, which binds only to GM1. The two molecules also have many immunologic differences, as demonstrated by immunodiffusion studies, against LT-associated E. coli diarrhea in volunteers receiving B-subunit whole whole-cell cholera vaccine. LT and CT induce different helper T-cell responses. When used as a mucosal adjuvant, CT selectively induces in some cases Th2-type cells in Peyers patches and spleens as manifested by production of interleukins 4 and 5, but not interleukin 2 or gamma interferon; while LT induces both Th1 and Th2 cells and predominantly antigen-specific IgA responses. Taken together, these findings demonstrate that LT and CT are unique molecules, despite their apparent structural similarities. Such differential

behavior makes the ability to activate LT so that it has potency similar to CT useful in manipulating the type of immune response produced to both the toxin itself and to antigens for which LT can be used as an adjuvant. It may also be possible that genetically altered toxoids such as mutants of the trypsin cleavage site may be active by transcutaneous immunication. Such a mutant toxin may be useful as it avoids the risks associated with ingestion or inhaling native toxins.

In a similar manner, PT may be activated to enhance its adjuvant and antigen activities. The S1 subunit of the hexameric PT protein contains the ADP-ribosyltransferase activity while the remaining subunits constitute the B domain. Similar to LT, PT has both trypsin cleavage sites and disulphide binding sites that play a role in association of the S1 subunit with the B oligomer. It is conceivable that activation by trypsin cleavage, disruption of the disulphide bond or both may enhance the adjuvant and antigen activities of PT in the context of transcutaneous immunization. Activation may also take the form of targeting, achieved by disruption of the hexamer into subunits. For example, the PT subunit S3 binds exclusively to the glycolipids of monocytes and could be used to target Langerhans cells in the skin.

Activation of the antigen or adjuvant could be extended to the concept of transcutaneous immunization using DNA by production of a fusion protein comprised of antigen and adjuvant domains. By this method a plasmid encoding an ADP-ribosylating exotoxin such as CT or LT and constructed to express a separate antigen such as a malaria or HIV antigen simultaneously could be placed on the skin in a hydrating solution or occlusive patch, and then taken up by Langerhans cells. Expression of the an ADP-ribosylating exotoxin component of the fusions protein such as CT or LT could activate the Langerhans cell, causing it to migrate and present antigen in the lymph node and therby induce an immune response to the encoded antigen. Another embodiment could include the conjugation of an adjuvant with a plasmid; an Fc portion of IgG to a plasmid to target APCs. A similar immunization could be achieved using separate plasmids for expressing an ADP-ribosylating exotoxin such as CT or LT and another for expressing the antigen such as a malaria or HIV antigen. It is conceivable that multiple genes on a single construct for multiple antigens could be used or multiple plasmids could be used to simultaneously deliver antigens for multivalent immunization. Plasmids encoding other molecules or compounds such as chemokines (e.g., defensins 1 or 2, RANTES, MIP1-α, MIP-2,

interleukin-8) or a cytokine (e.g., interleukin-1β, -2, -6, -10 or -12; γ-interferon; tumor necrosis factor-α; or granulocyte-monocyte-colony stimulating factor) (reviewed in Nohria and Rubin, 1994), a heat shock protein or a derivative, a derivative of Leishmania major LeIF (Skeiky et al., 1995), cholera toxin toxin B, a lipopolysaccharide (LPS) derivative (e.g., lipid A or monophosphoryl lipid A), or superantigen (Saloga et al., 1996), or other ADP-ribosylating exotoxins might be delivered with protein antigens.

Other means of activating the transcutaneous adjuvants may be effective, such as adding detergents and phospholipid to the formulation to enhance CT activity by ADP-ribosylation factor (see, for example, Spangler, 1992).

For immunization using adjuvant or antigen activation, modification of the adjuvant or antigen component of the formulation may reduce its effectiveness in parenteral immunization without destroying the utility of the formulation in transcutaneous immunization when the adjuvant and/or antigen is activated. Undesirable properties (e.g., toxicity, allergic reactivity, other side effects) of the adjuvant or antigen in the formulation may be reduced by modification without destroying its effectiveness in transcutaneous immunization. Activation of such modified adjuvant or antigen may involve, for example, removal of a reversible chemical modification (e.g., proteolysis) or a coating which reversibly isolates a component of the formulation from the immune system (i.e., an encapsulated formulation). Alternatively, the adjuvant and/or antigen comprising the formulation may be encapsulated in a particle (e.g., microspheres, nanoparticles). Phagocytosis of a particle may, by itself, enhance activation of an antigen presenting cell by upregulating expression of major histocompatibility antigens and/or costimulatory molecules (e.g., MHC class II, B7-2).

ANTIGEN

Antigen of the invention may be expressed by recombinant means, preferably as a fusion with an affinity or epitope tag (Summers and Smith, 1987; Goeddel, 1990; Ausubel et al., 1996); chemical synthesis of an oligopeptide, either free or conjugated to carrier proteins, may be used to obtain antigen of the invention (Bodanszky, 1993; Wisdom, 1994). Oligopeptides are considered a type of polypeptide. Oligopeptide lengths of 6 residues to 20 residues are preferred. Polypeptides may also be synthesized as branched structures such as those disclosed in U.S. Pat. Nos. 5,229,490 and 5,390,111. Antigenic polypeptides include, for example, synthetic or recombinant B-cell and T-cell epitopes, universal T-cell epitopes, and mixed T-cell epitopes from one organism or disease and B-cell epitopes from another. Antigen obtained through recombinant means or peptide synthesis, as well as antigen of the invention obtained from natural sources or extracts, may be purified by means of the antigen's physical and chemical characteristics, preferably by fractionation or chromatography (Janson and Ryden, 1989; Deutscher, 1990; Scopes, 1993). A multivalent antigen formulation may be used to induce an immune response to more than one antigen at the same time. Conjugates may be used to induce an immune response to multiple antigens, to boost the immune response, or both. Additionally, toxins may be boosted by the use of toxoids, or toxoids boosted by the use of toxins. Transcutaneous immunization may be used to boost responses induced initially by other routes of immunization such as by oral, nasal or parenteral routes. Antigen includes, for example, toxins, toxoids, subunits thereof, or combinations thereof (e.g., cholera toxin, tetanus toxoid); additionally, toxins, toxoids, subunits thereof, or combinations thereof may act as both antigen and adjuvant.

Antigen may be solubilized in a buffer. Suitable buffers include, but are not limited to, phosphate buffered saline Ca^{++}/Mg^{++} free (PBS), normal saline (150 mM NaCl in water), and Tris buffer. Glycerol may be a suitable non-aqueous buffer for use in the present invention. Antigen may also be in suspension. The detergent may be left in the immunizing soluton to enhance penetration.

Hydrophobic antigen can be solubilized in a detergent, for example a polypeptide containing a membrane-spanning domain. Furthermore, for formulations containing liposomes, an antigen in a detergent solution (e.g., a cell membrane

extract) may be mixed with lipids, and liposomes then may be formed by removal of the detergent by dilution, dialysis, or column chromatography. See, Gregoriadis (1993). Certain antigens such as, for example, those from a virus (e.g., hepatitis A) need not be soluble per se, but can be incorporated directly into a lipid membrane (e.g., a virosome as described by Morein and Simons, 1985), in a suspension of virion alone, or suspensions of microspheres nanoparticles or heat-inactivated bacteria which may be taken up by and activate antigen presenting cells (e.g., opsonization).

Plotkin and Mortimer (1994) provide antigens which can be used to vaccinate animals or humans to induce an immune response specific for particular pathogens, as well as methods of preparing antigen, determining a suitable dose of antigen, assaying for induction of an immune response, and treating infection by a pathogen (e.g., bacterium, virus, fungus, or parasite).

Bacteria include, for example: anthrax, campylobacter, cholera, clostridia, diphtheria, enterotoxigenic E. coli, giardia, gonococcus, Helicobacter pylori or urease produced by H. pylori (Lee and Chen, 1994), Hemophilus influenza B, Hemophilus influenza non-typable, meningococcus, mycobacterium, pertussis, pneumococcus, salmonella, shigella, staphylococcus, Streptococcus B, tetanus, Vibrio cholerae, Borrelia burgdorfi and Yersinia; and products thereof.

Viruses include, for example: adenovirus, dengue serotypes 1 to 4 (Delenda et al., 1994; Fonseca et al., 1994; Smucny et al., 1995), ebola (Jahrling et al., 1996), enterovirus, hanta virus, hepatitis serotypes A to E (Blum, 1995; Katkov, 1996; Lieberman and Greenberg, 1996; Mast, 1996; Shafara et al., 1995; Smedila et al., 1994; U.S. Pat. Nos. 5,314,808 and 5,436,126), herpes simplex virus 1 or 2, human immunodeficiency virus (Deprez et al., 1996), human papilloma virus, influenza, measles, Norwalk, Japanese equine encephalitis, papilloma virus, parvovirus B19, polio, rabies, respiratory syncytial virus, rotavirus, rubella, rubeola, St. Louis encephalitis, vaccinia, vaccinia constructs containing genes coding for other antigens such as malaria antigens, varicella, and yellow fever; and products thereof.

Parasites include, for example: Entamoeba histolytica (Zhang et al., 1995); Plasmodium (Bathurst et al., 1993; Chang et al., 1989, 1992, 1994; Fries et al., 1992a, 1992b; Herrington et al., 1991; Khusmith et al., 1991; Malik et al., 1991; Migliorini et al., 1993; Pessi et al., 1991; Tam, 1988; Vreden et al., 1991; White et al., 1993;

Wiesmueller et al., 1991), Leishmania (Frankenburg et al., 1996), and the Helminthes; and products thereof.

Other viruses which can be used in the present invention are disclosed in Gordon, 1997 and include, for example, Adenovirus (respiratory disease), Coronavirus (respiratory and enteric disease), Cytomegalovirus (mononucleosis), Dengue virus (dengue fever, shock syndrome), Epstein-Barr virus (mononucleosis, Burkitt's lymphoma), Hepatitis A, B and C virus (liver disease), Herpes simplex virus type 1 (encephalitis, stomatitis), Herpes simplex virus type 2 (genital lesions), Human herpesvirus-6 (unknown, possibly Kaposi's sarcoma), Human immunodeficiency virus types 1 and 2 (acquired immunodeficiency syndrome-AIDS), Human T-cell lymphotropic virus type 1 (T-cell leukemia), Influenza A, B, and C (respiratory disease), Japanese encephalitis virus (pneumonia, encephalopathy), Measles virus (subacute sclerosing panencephalitis), Mumps virus (meningitis, encephalitis), Papillomavirus (warts, cervical carcinoma), Parvovirus (respiratory disease, anemia), Poliovirus (paralysis), Polyomavirus JC (multifocal leukoencephalopathy), Polyomavirus BK (hemorrhagic cystitis), Rabies virus (nerve dysfunction), Respiratory syncytial virus (respiratory disease), Rhinovirus (common cold), Rotavirus (diarrhea), Rubella virus (fetal malformations), Vaccinia virus (generalized infection), Yellow fever virus (jaundice, renal and hepatic failure), Varicella zoster virus (chickenpox).

Other bacteria which can be used in the present invention are disclosed in Gordon, 1997 and include, for example, Bacillus anthracis (anthrax), Bordetella pertussis (whooping cough), Borrelia burgdorferi (lyme disease), Campylobacter jejuni (gastroenteritis), Chlamydia trachomatis (pelvic inflammatory disease, blindness), Clostridium botulinum (botulism), Corynebacterium dipththeriae (diphtheria), Escherichia coli (diarrhea, urinary tract infections), Haemophilus influenzae (pneumonia), Helicobacter pylori (gastritis, duodenal ulcer), Legionella pneumophila (Legionnaires' disease), Listeria monocytogenes (meningitis, sepsis), Mycobacterium leprae (leprosy), Mycobacterium tuberculosis (tuberculosis), Neisseria gonorrhoeae (gonorrhea), Neisseria meningitidis (sepsis, meningitis), Pseudomonas aeruginosa (nosocomial infections), Pseudomonas aeruginosa (nosocomial infections), Rickettsia (Rocky Mountain spotted fever), Salmonella (typhoid fever, gastroenteritis), Shigella (dysentery), Staphylococcus aureus

(impetigo, toxic shock syndrome), Streptococcus pneumoniae (pneumonia, otitis media), Streptococcus pyogenes (Rheumatic fever, pharyngitis), Treponema pallidum (syphilis), Vibrio cholerae (cholera), Yersinia pestis (bubonic plague).

Other parasites which can be used in the present invention are disclosed in Gordon, 1997 and include, for example, African trypanosomes (trypanosomiasis), Entamoeba histolytica (amebic dysentery), Giardia lamblia (diarrheal disease), Leishmania (lesions of the spleen, tropical sores), Plasmodium (malaria), Microfilariae (filariasis), Schistosomes (schistosomiasis), Toxoplasma gondii (toxoplasmosis), Trichomonas vaginalis (vaginitis), Trypanosoma cruzi (Chagas disease).

Fungi which can be used in the present invention are disclosed in Gordon, 1997 and include, for example, Candida albicans (mucosal infections), Histoplasma (lung, lymph node infections), Pneumocystis carinii (pneumonia in AIDS), Aspergillus fumigatis (aspergillosis).

ADJUVANT

The formulation also contains an adjuvant, although a single molecule may contain both adjuvant and antigen properties (e.g., cholera toxin) (Elson and Dertzbaugh, 1994). Adjuvants are substances that are used to specifically or non-specifically potentiate an antigen-specific immune response. Usually, the adjuvant and the formulation are mixed prior to presentation of the antigen but, alternatively, they may be separately presented within a short interval of time.

Adjuvants are usually products of bacteria, parasites or even viruses or bacteria, but may be derived from other natural or synthetic sources. Adjuvants include, for example, an oil emulsion (e.g., complete or incomplete Freund's adjuvant), a chemokine (e.g., defensins 1 or 2, RANTES, MIP1-α, MIP-2, interleukin-8) or a cytokine (e.g., interleukin-1β, -2, -6, -10 or -12; γ-interferon; tumor necrosis factor-α; or granulocyte-monocyte-colony stimulating factor) (reviewed in Nohria and Rubin, 1994), a muramyl dipeptide derivative (e.g., murabutide, threonyl-MDP or muramyl tripeptide), a heat shock protein or a derivative, a derivative of Leishmania major LeIF (Skeiky et al., 1995), cholera toxin or cholera toxin B, bARES, a lipopolysaccharide (LPS) derivative (e.g., lipid A or monophosphoryl lipid A , synthetic lipid A analogues), or superantigen (Saloga et al., 1996) block copolymers or

other polymers known in the art. Also, see Richards et al. (1995) for other adjuvants useful in immunization.

An adjuvant may be chosen to preferentially induce antibody or cellular effectors, specific antibody isotypes (e.g., IgM, IgD, IgA1, IgA2, secretory IgA, IgE, IgG1, IgG2, IgG3, and/or IgG4), or specific T-cell subsets (e.g., CTL, Th1, Th2 and/or T_{DTH}) (Munoz et al., 1990; Glenn et al., 1995).

CpGs are among a class of structures which have patterns allowing the immune system to recognize their pathogenic origins to stimulate the innate immune response leading to adaptive immune responses (Medzhitov and Janeway, 1997). These structures are called pathogen-associated molecular patterns (PAMPs) and include lipopolysaccharides, teichoic acids, unmethylated CpG motifs, double stranded RNA and mannins, for example.

PAMPs induce endogenous danger signals that can enhance the immune response, act as costimulators of T-cell function and control the effector function. The ability of PAMPs to induce these responses play a role in their potential as adjuvants and their targets are APCs such as macrophages and dendritic cells. The antigen presenting cells of the skin could likewise be stimulated by PAMPs transmitted through the skin. For example, Langerhans cells, a type of dendritic cell, could be activated by a PAMP in solution on the skin with a transcutaneously poorly immunogenic molecule and be induced to migrate and present this poorly immunogenic molecule to T-cells in the lymph node, inducing an antibody response to the poorly immunogenic molecule. PAMPs could also be used in conjunction with other skin adjuvants such as cholera toixn to induce different costimulatory molecules and control different effector functions to guide the immune response, for example from a Th2 to a Th1 response.

Cholera toxin is a bacterial exotoxin from the family of ADP-ribsoylating exotoxins (referred to as bAREs). Most bAREs are organized as A:B dimer with a binding B subunit and an A subunit containing the ADP-ribosyltransferase. Such toxins include diphtheria, Pseudomonas exotoxin A, cholera toxin (CT), E. coli heat-labile enterotoxin (LT), pertussis toxin (PT), C. botulinum toxin C2, C. botulinum toxin C3, C. limosum exoenzyme, B. cereus exoenzyme, Pseudomonas exotoxin S, Staphylococcus aureus EDIN, and B. sphaericus toxin.

Cholera toxin is an example of a bARE that is organized with A and B subunits. The B subunit is the binding subunit and consists of a B-subunit pentamer which is non-covalently bound to the A subunit. The B-subunit pentamer is arranged in a symmetrical doughnut-shaped structure that binds to GM1-ganglioside on the target cell. The A subunit serves to ADP ribosylate the alpha subunit of a subset of the hetero trimeric GTP proteins (G proteins) including the Gs protein which results in the elevated intracellular levels of cyclic AMP. This stimulates release of ions and fluid from intestinal cells in the case of cholera.

Cholera toxin (CT) and its B subunit (CTB) have adjuvant properties when used as either an intramuscular or oral immunogen (Elson and Dertzbaugh, 1994; Trach et al., 1997). Heat-labile enterotoxin from E. coli (LT) is 75-77% homologous at the amino acid level with CT and possesses similar binding properties; it also appears to bind the GM1-ganglioside receptor in the gut and has similar ADP-ribosylating exotoxin activities. Another bARE, Pseudomonas exotoxin A (ETA), binds to the α2-macroglobulin receptor-low density lipoprotein receptor-related protein (Kounnas et al., 1992). bAREs are reviewed by Krueger and Barbieri (1995). CT, CTB, LT, ETA and PT, despite having different cellular binding sites, are potent adjuvants for transcutaneous immunization, inducing high levels of IgG antibodies but not IgE antibodies. CTB without CT can also induce high levels of IgG antibodies. Thus, both bAREs and a derivative thereof can effectively immunize when epicutaneouly applied to the skin in a simple solution.

All licensed vaccines require a level of antibody for approval – no other immune component such as T-cell proliferation is used. Protection against the life-threatening infections diphtheria, pertussis, and tetanus (DPT) can be achieved by inducing high levels of circulating anti-toxin antibodies. Pertussis may be an exception in that some investigators feel that antibodies directed to other portions of the invading organism are necessary for protection, although this is controversial (see Schneerson et al., 1996) and most new generation acellular pertussis vaccines have PT as a component of the vaccine (Krueger and Barbieri, 1995). The pathologies in the diseases caused by DPT are directly related to the effects of their toxins and anti-toxin antibodies most certainly play a role in protection (Schneerson et al., 1996).

In general, toxins can be chemically inactivated to form toxoids which are less toxic but remain immunogenic. We envision that the one embodiment of

transcutaneous immunization system will use toxin-based immunogens and adjuvants to achieve anti-toxin levels adequate for protection against these diseases. The anti-toxin antibodies may be induced through immunization with the toxins, or genetically-detoxified toxoids themselves, or with toxoids and adjuvants such as CT. Genetically toxoided toxins which have altered ADP-ribosylating exotoxin activity, or trypsin cleavage site mutations or other mutations are envisioned to be especially useful as non-toxic activators of antigen presenting cells used in transcutaneous immunization. Mutants based on inactivating the catalytic activity of the ADP-ribosyl transferase by genetic deletion retain the binding capabilities, but lack the toxicity, of the natural toxins. This approach is described by Burnette et al. (1994), Rappuoli et al. (1995), and Rappuoli et al. (1996). Such genetically toxoided exotoxins could be useful for transcutaneous immunization system in that they would not create a safety concern as the toxoids would not be considered toxic. There are other genetically altered toxins which have, for example, deletions of the trysin cleavage site and use both non-toxic and immunogeneric on the skin. However, activation through a technique such as trypsin cleavage would be expected to enhance the adjuvant qualities of LT through the skin which is lacking inherent trypsin enzymes. Additionally, several techniques exist to chemically toxoid toxins which can address the same problem (Schneerson et al., 1996). These techniques could be important for certain applications, especially pediatric applications, in which ingested toxins (e.g., diphtheria toxin) might possibly create adverse reactions.

Optionally, an activator of Langerhans cells may be used as an adjuvant. Examples of such activators include: an inducer of heat shock protein; contact sensitizer (e.g., trinitrochlorobenzene, dinitrofluorobenzene, nitrogen mustard, pentadecylcatechol); toxin (e.g., Shiga toxin, Staph enterotoxin B); lipopolysaccharide, lipid A, or derivatives thereof; bacterial DNA (Stacey et al., 1996); cytokine (e.g., tumor necrosis factor-α, interleukin-1β, -10, -12); calcium ions in solution; calcium ionophores, and chemokine (e.g., defensins 1 or 2, RANTES, MIP-1α, MIP-2, interleukin-8).

If an immunizing antigen has sufficient Langerhans cell activating capabilities then a separate adjuvant may not be required, as in the case of CT which is both antigen and adjuvant. It is envisioned that whole cell preparations, live viruses, attenuated viruses, DNA plasmids, and bacterial DNA could be sufficient to

immunize transcutaneously then an adjuvant is present. It may be possible to use low concentrations of contact sensitizers or other activators of Langerhans cells to induce an immune response without inducing skin lesions.

PRACTICAL ASPECTS OF TRANSCUTANEOUS IMMUNIZATION

Efficient immunization can be achieved with the present invention because transcutaneous delivery of antigen may target the Langerhans cell. These cells are found in abundance in the skin and are efficient antigen presenting cells leading to T-cell memory and potent immune responses. Because of the presence of large numbers of Langerhans cells in the skin, the efficiency of transcutaneous delivery may be related to the surface area exposed to antigen and adjuvant. In fact, the reason that transcutaneous immunization is so efficient may be that it targets a larger number of these efficient antigen presenting cells than intramuscular immunization.

We envision the present invention will enhance access to immunization, while inducing a potent immune response. Because transcutaneous immunization does not involve physical penetration of the skin and the complications and difficulties thereof, the requirements of trained personnel, sterile technique, and sterile equipment are reduced. Furthermore, the barriers to immunization at multiple sites or to multiple immunizations are diminished. Immunization by a single application of the formulation is also envisioned, but boosting is generally needed. Needle free immunication is a priority for the World Health Organization (WHO) because of the reuse of needles which causes needle-borne disease.

Immunization may be achieved using epicutaneous application of a simple solution of antigen and adjuvant impregnated in gauze under an occlusive patch, or by using other patch technologies; creams, gels, immersion, ointments and sprays are other possible methods of application. The immunization could be given by untrained personnel, and is amenable to self-application. Large-scale field immunization could occur given the easy accessibility to immunization. Additionally, a simple immunization procedure would improve access to immunization by pediatric patients and the elderly, and populations in Third World countries.

For previous vaccines, their formulations were injected through the skin with needles. Injection of vaccines using needles carries certain drawbacks including the

need for sterile needles and syringes, trained medical personnel to administer the vaccine, discomfort from the injection, and potential complications brought about by puncturing the skin with the needle. Immunization through the skin without the use of needles (i.e., transcutaneous immunization) represents a major advance for vaccine delivery by avoiding the aforementioned drawbacks.

Moreover, transcutaneous immunization may be superior to immunization using needles as more immune cells would be targeted by the use of several locations targeting large surface areas of skin. A therapeutically effective amount of antigen sufficient to induce an immune response may be delivered transcutaneously either at a single cutaneous location, or over an area of intact skin covering multiple draining lymph node fields (e.g., cervical, axillary, inguinal, epitrochelear, popliteal, those of the abdomen and thorax). Such locations close to numerous different lymphatic nodes at locations all over the body will provide a more widespread stimulus to the immune system than when a small amount of antigen is injected at a single location by intradermal subcutaneous or intramuscular injection.

Antigen passing through or into the skin may encounter antigen presenting cells which process the antigen in a way that induces an immune response. Multiple immunization sites may recruit a greater number of antigen presenting cells and the larger population of antigen presenting cells that were recruited would result in greater induction of the immune response. It is conceivable that absorption through the skin may deliver antigen to phagocytic cells of the skin such as, for example, dermal dendritic cells, macrophages, and other skin antigen presenting cells; antigen may also be delivered to phagocytic cells of the liver, spleen, and bone marrow that are known to serve as the antigen presenting cells through the blood stream or lymphatic system. Langerhans cells, dendritic cells, and macrophages may be specifically targeted using Fc receptor conjugated to or recombinantly produced as a protein fusion with adjuvant; also, complement receptors (C3, C5) may be conjugated to or recombinantly produced as a protein fusion with protein A or protein G to target surface immunoglobulin of B cells. The result would be targeted distribution of antigen to antigen presenting cells to a degree that is rarely, if ever achieved, by current immunization practices.

The transcutaneous immunization system may be applied directly to the skin and allowed to air dry; rubbed into the skin or scalp; held in place with a dressing,

patch, or absorbent material; immersion; otherwise held by a device such as a stocking, slipper, glove, or shirt; or sprayed onto the skin to maximize contact with the skin. The formulation may be applied in an absorbant dressing or gauze. The formulation may be covered with an occlusive dressing such as, for example, AQUAPHOR (an emulsion of petrolatum, mineral oil, mineral wax, wool wax, panthenol, bisabol, and glycerin from Beiersdorf, Inc.), plastic film, COMFEEL (Coloplast) or vaseline; or a non-occlusive dressing such as, for example, DUODERM (3M) or OPSITE (Smith & Napheu). An occlusive dressing completely excludes the passage of water. The formulation may be applied to single or multiple sites, to single or multiple limbs, or to large surface areas of the skin by complete immersion. The formulation may be applied directly to the skin.

Genetic immunization has been described in U.S. Pat. Nos. 5,589,466, 5,593,972, and 5,703,055. The nucleic acid(s) contained in the formulation may encode the antigen, the adjuvant, or both. It would generally be expected that the immune response would be enhanced by the coadministration of an adjuvant, for example, CT, LT or CpGs to the nucleic acid encoding for the antigen. The nucleic acid may or may not be capable of replication; it may be non-integrating and non-infectious. For example, the nucleic acid may encode a fusion polypeptide comprising antigen and a ubiquitin domain to direct the immune response to a class I restricted response. The nucleic acid may further comprise a regulatory region (e.g., promoter, enhancer, silencer, transcription initiation and termination sites, RNA splice acceptor and donor sites, polyadenylation signal, internal ribosome binding site, translation initiation and termination sites) operably linked to the sequence encoding the antigen. The nucleic acid may be complexed with an agent that promotes transfection such as cationic lipid, calcium phosphate, DEAE-dextran, polybrene-DMSO, or a combination thereof; also, immune cells can be targeted by conjugation of DNA to Fc receptor or protein A/G, or encapsulating DNA in an agent linked to Fc receptor or protein A/G. The nucleic acid may comprise regions derived from viral genomes. Such materials and techniques are described by Kriegler (1990) and Murray (1991).

An immune response may comprise humoral (i.e., antigen-specific antibody) and/or cellular (i.e., antigen-specific lymphocytes such as B cells, $CD4^+$ T cells, $CD8^+$ T cells, CTL, Th1 cells, Th2 cells, and/or T_{DTH} cells) effector arms. Moreover, the

immune response may comprise NK cells that mediate antibody-dependent cell-mediated cytotoxicity (ADCC).

The immune response induced by the formulation of the invention may include the elicitation of antigen-specific antibodies and/or cytotoxic lymphocytes (CTL, reviewed in Alving and Wassef, 1994). Antibody can be detected by immunoassay techniques, and the detection of various isotypes (e.g., IgM, IgD, IgA1, IgA2, secretory IgA, IgE, IgG1, IgG2, IgG3, or IgG4) may be expected. An immune response can also be detected by a neutralizing assay. Antibodies are protective proteins produced by B lymphocytes. They are highly specific, generally targeting one epitope of an antigen. Often, antibodies play a role in protection against disease by specifically reacting with antigens derived from the pathogens causing the disease.

CTLs are particular protective immune cells produced to protect against infection by a pathogen. They are also highly specific. Immunization may induce CTLs specific for the antigen, such as a synthetic oligopeptide based on a malaria protein, in association with self-major histocompatibility antigen. CTLs induced by immunization with the transcutaneous delivery system may kill pathogen infected cells. Immunization may also produce a memory response as indicated by boosting responses in antibodies and CTLs, lymphocyte proliferation by culture of lymphocytes stimulated with the antigen, and delayed type hypersensitivity responses to intradermal skin challenge of the antigen alone.

In a viral neutralization assay, serial dilutions of sera are added to host cells which are then observed for infection after challenge with infectious virus. Alternatively, serial dilutions of sera may be incubated with infectious titers of virus prior to innoculation of an animal, and the innoculated animals are then observed for signs of infection.

The transcutaneous immunization system of the invention may be evaluated using challenge models in either animals or humans, which evaluate the ability of immunization with the antigen to protect the subject from disease. Such protection would demonstrate an antigen-specific immune response. In lieu of challenge, for example achieving anti-diphtheria antibody titers of 5 IU/ml or greater is generally assumed to indicate optimum protection and serves as a surrogate marker for protection (Plotkin and Mortimer, 1994).

Vaccination has also been used as a treatment for cancer and autoimmune disease. For example, vaccination with a tumor antigen (e.g., prostate specific antigen) may induce an immune response in the form of antibodies, CTLs and lymphocyte proliferation which allows the body's immune system to recognize and kill tumor cells. Tumor antigens useful for vaccination have been described for melanoma (U.S. Pat. Nos. 5,102,663, 5,141,742, and 5,262,177), prostate carcinoma (U.S. Pat. No. 5,538,866), and lymphoma (U.S. Pat. Nos. 4,816,249, 5,068,177, and 5,227,159). Vaccination with T-cell receptor oligopeptide may induce an immune response that halts progression of autoimmune disease (U.S. Pat. Nos. 5,612,035 and 5,614,192; Antel et al., 1996; Vandenbark et al., 1996). U.S. Pat. No. 5,552,300 also describes antigens suitable for treating autoimmune disease.

The following is meant to be illustrative of the present invention; however, the practice of the invention is not limited or restricted in any way by the examples.

EXAMPLES

Immunization procedure. Twenty four hours prior to immunization, the back of the mouse is shaved from the distal aspect of the scapula to 0.5 cm above the base of the tail. In the case of C57BL/6 mice, the animals are lightly anesthetized (40 mg/kg ketamine : 4 mg/kg xylazine mixture in saline) prior to shaving. On the day of immunization the animals are immunized with .04 ml of an anesthesia mixture (2.3 mL sterile saline (Sigma): 5 mL ketamine (100 mg/mL, Parke-Davis) : 0.5 mL xylazine (100 mg/mL, Phoenix Pharmaceuticals)) which delivers a final dose of approximately 110 mg/kg ketamine and 11 mg/kg xylazine. For procedures requiring alcohol swabbing, the back is wiped 10x (5x sweeping up the back towards the head, flip over alcohol pad and sweep back 5x more) using an isopropyl pad. The alcohol is allowed to evaporate for 5 minutes. Hydration of the back is accomplished by gently rubbing the back with a sterile water-saturated gauze pad so as to form a pool of water on the back. After a 5 minute hydration period, the back is blotted dry with a dry gauze pad. Next, antigen - generally $\leq$ 100 µg of antigen and adjuvant in 100 µl final volume, is applied to the back using a pipette and tip and left on the skin for 60 to 120 minutes. After the defined immunization period has been reached, any excess solution in the immunized area is blotted with cotton gauze. The animals are then rinsed animals under a slow steady stream of lukewarm tap water for 10 seconds to remove any excess antigen, blotted dry and the rinsing procedure repeated. The cages

are then placed onto the heating pads until they are fully recovered from the anesthesia.

Measurement of Human anti-LT Antibody Titers. Anti-LT IgG titers were determined as previously described (Svennerholm A-M., Holmgren, J., Black, R., Levine, M. & Merson, M. Serologic differentation between antitoxin responses to infection with Vibrio cholerae and enterotoxin-producing Escherichia coli. *J. Infect. Dis.* **147**, 541-522 (1983). 96 well (Type-Russell) plates were coated overnight with monosialoganglioside-G_{M1} (Sigma, St. Louis, MO) of LT (Sigma) , blocked with 5% dry milk in PBS-0.05% Tween. Responses were detected using goat anti-human IgG(γ)-HRP (Kirkegaard and Perry, Gaithersburg, MD., and 2,2'-azino-di[3-ethylbenzthiazoline sulfonate (Kirkegaard and Perry)as substrate and plates were read at 405 nm. Results are reported in ELISA units (EU) which are defined as the inverse dilution of sample which yields an OD of 1.0. Anti-LT IgA was determined in the same manner as anti-LT IgG except that goat anti-human IgA(α)-HRP (Kirkegaard and Perry) was used as secondary antibody and ODs were plotted against a standard IgA curve yielding results expressed in ng/ml. The standard IgA curve and total serum IgA were determined by using unlabeled goat anti-human IgA (Kirkegaard and Perry) followed by blocking as above and then application of serial dilutions of IgA standard

Example 1.

Swabbing the skin with a treated or untreated swab is thought to physically and chemically remove a small portion of the stratum corneum and thus enhance skin penetration. Swabs can be made of materials such as, for example, cotton, nylon, rayon and polyethylene. Alcohol swabbing is thought to remove a small portion of the stratum corneum and acts both as a physical means and chemical means of penetration enhancement. In the example above, the enhancement of the immune response to transcutaneous immunization can be seen with this penetration enhancement method. BALB/c mice 6 to 8 weeks of age were anesthetized and shaved as described in the "immunization procedure". Twenty four hours later, the backs of the animals were either wiped with a gauze pad saturated in water "water" or wiped for approximately 10 seconds with an alcohol prep pad containing 70% isopropyl alcohol "isopropanol". The alcohol was allowed to evaporate for approximately 5 minutes. The excess water was removed from the backs of the

"water" group by blotting. All animals were then treated with 20 μg of CT (100 μl of a 0.2 mg/ml solution). Removal of excess antigen was conducted as described in the "immunization procedure."

The anti-CT antibody titers were determined using ELISA as described above for "ELISA IgG (H+L)" 3 weeks after a single immunization. The results are shown in Table 1. While CT was clearly immunogenic in both groups, the group treated with the alcohol prep pads exhibited a geometric mean titer that was 6 fold higher and the individual titers were more consistent than the "water" animals. Thus it appears that chemical and physical disruption of the skin surface with alcohol swabs enhances delivery of antigen by the transcutaneous route.

Table 1. Enhancement of transcutaneous immunization by chemical penetration enhancement: Anti-CT titers in mice that had the skin treated with an alcohol prep pad before application of the antigen.

		anti-CT IgG (H+L) ELISA units	
Animal #	treatment	prebleed	week 3
7146	water		1275
7147	water		69
7148	water		7420
7149	water		6025
7150	water		388
geometric mean			1088
pooled prebleed		7	
7161	isopropanol		3100
7162	isopropanol		14797
7163	isopropanol		6670
7164	isopropanol		7426
7165	isopropanol		7024
geometric mean			6928
pooled prebleed		7	

Example 2.

To assess whether chemical penetration enhancement alone might augment transcutaneous immunization a detergent was used on the skin. BALB/c mice 6 to 8 weeks of age were anesthetized and shaved as described in the "immunization procedure." Twenty-four hours later, the backs of the "water" group were wiped with a gauze pad saturated in water and a pool of water was placed on the back. Approximately 5 minutes later, any excess water was removed and 25 μg of CT (50 μl of a 0.5 mg/ml solution) was applied to the back. Alternatively, 24 hours after shaving, the backs of the "5% SDS" group were treated by dripping 300 μl of 5% SDS (Sodium Dodecyl Sulfate – a 1 to 1 mixture of deionized water and commercial stock of 10% SDS), a detergent, for approximately 12 minutes followed by blotting off any excess SDS with a dry gauze pad. SDS can be applied to the skin in a carrier such as, for example, a pad and then any excess SDS can be removed with a dry gauze pad. Thereafter the animals were hydrated and immunized as per the "water" group. Removal of excess antigen was conducted as described in the "immunization procedure."

The anti-CT antibody titers were determined using ELISA as described above for "ELISA IgG (H+L)" 2 weeks after a single immunization. The results are shown in tables 2a and 2b. While CT was clearly immunogenic in both groups, the geometric mean titer in the 5% SDS treated group approximately 2 fold higher and the titers were more consistent among the latter animals as compared with the "water" animals. Thus it appears that chemical disruption of the skin surface with detergent (5% SDS) enhances delivery of antigen by the transcutaneous route.

Table 2a. Enhancement of transcutaneous immunization by chemical penetration enhancement: Anti-CT titers in mice that had the skin treated with detergent (5% SDS) before application of the antigen.

		anti-CT IgG (H+L) ELISA units	
Animal #	treatment	prebleed	week 2
546	water		4629
547	water		3154
548	water		7288
549	water		1719
550	water		11779
geometric mean			3678
pooled prebleed		5	
596	5% SDS		6945
597	5% SDS		2244
598	5% SDS		8604
599	5% SDS		7093
600	5% SDS		12583
geometric mean			5553
pooled prebleed		1	

Table 2b. Enhancement of transcutaneous immunization by chemical penetration enhancement: Anti-CT titers in mice that had the skin treated with detergent (5% SDS) before application of the antigen.

		anti-CT IgG (H+L) ELISA units	
Animal #	treatment	prebleed	week 3
546	water		22525
547	water		8939
548	water		11885
549	water		5121
550	water		37770
geometric mean			10521
pooled prebleed		11	
596	5% SDS		102387
597	5% SDS		6597
598	5% SDS		47245
599	5% SDS		45565
600	5% SDS		38413
geometric mean			34725
pooled prebleed		6	

Example 3.

Another form of chemical penetration enhancement, a depilatory (such as, for example, calcium hydroxide or the like) is widely used in dermatologic experiments and was shown to enhance transcutaneous immunization. BALB/c mice 6 to 8 weeks of age were anesthetized and shaved as described in the "immunization procedure." Twenty-four hours later, the backs of the "water" group were wiped with a gauze pad saturated in water and a pool of water was placed on the back. Approximately 5 minutes later, any excess water was removed and 25 μg of CT (50 μl of a 0.5 mg/ml solution) was applied to the back. Alternatively, twenty-four hours after shaving, the backs of the "nair©" group were treated with 100 μl of nair© cream for approximately 12 minutes followed by wiping off of the formulation with a gauze pad saturated in water. Such treatment can continue for from about 0.1 to 30 minutes preferably about 20 minutes and more preferably about 12 minutes. Thereafter the animals were hydrated and immunized as per the "water" group. Removal of excess antigen was conducted as described in the "immunization procedure."

The anti-CT antibody titers were determined using ELISA as described above for "ELISA IgG (H+L)" 2 weeks after a single immunization. The results are shown in tables 3a and 3b. While CT was clearly immunogenic in both groups, the geometric mean titer in the nair treated group was 3 fold higher and the titers were more consistent among the latter animals as compared with the "water" animals. Thus it appears that chemical disruption of the skin surface with calcium hydroxide, the active ingredient in nair© cream, enhances delivery of antigen by the transcutaneous route.

Table 3a. Enhancement of transcutaneous immunization by chemical penetration enhancement: Anti-CT titers in mice that had the skin treated with calcium hydroxide (nair©) before application of the antigen.

		anti-CT IgG (H+L) ELISA units	
Animal #	treatment	prebleed	week 2
546	water		4629
547	water		3154
548	water		7288
549	water		1719
550	water		11779
geometric mean			3678
pooled prebleed		5	
581	nair©		17621
582	nair©		12261
583	nair©		7235
584	nair©		7545
585	nair©		5997
geometric mean			10421
pooled prebleed		4	

Table 3b. Enhancement of transcutaneous immunization by chemical penetration enhancement: Anti-CT titers in mice that had the skin treated with calcium hydroxide (nair©) before application of the antigen.

		anti-CT IgG (H+L) ELISA units	
Animal #	treatment	prebleed	Week 3
546	water		22525
547	water		8939
548	water		11885
549	water		5121
550	water		37770
geometric mean			10521
pooled prebleed		11	
581	nair©		34222
582	nair©		45674
583	nair©		50224
584	nair©		27270
585	nair©		21832
geometric mean			38251
pooled prebleed		15	

Example 4.

Further studies were conducted to evaluate the effect of chemical penetration enhancement using a keratinolytic formulation (such as a salicylate). BALB/c mice 6 to 8 weeks of age were anesthetized and shaved as described in the "immunization procedure." Twenty-four hours later, the backs of the "water" group were wiped with a gauze pad saturated in water and a pool of water was placed on the back. Approximately 5 minutes later, any excess water was removed and 25 μg of CT (50 μl of a 0.5 mg/ml solution) was applied to the back. Alternatively, twenty-four hours after shaving, the backs of the "salicylate/water" group were treated with a gauze pad saturated with a 10% salicylate suspension (1 tablet (325 mg) Certified brand aspirin dissolved in 3.25 ml deionized water). Such treatment can continue for from about 0.1 to 30 minutes preferably about 20 minutes and more preferably about 10 minutes. Approximately 10 minutes later any remaining solution was blotted off, the backs of the animals were hydrated with water for 5 minutes, followed by removal of excess water, and then topical application of 25 μg of CT. Removal of excess antigen was conducted as described in the "immunization procedure."

The anti-CT antibody titers were determined using ELISA as described above for "ELISA IgG (H+L)" 2 weeks after a single immunization. The results are shown in Table 4. While CT was clearly immunogenic in both groups, the geometric mean titer in the salicylate treated group was 4 fold higher and the titers were more consistent among the latter animals as compared with the "water" animals. Thus it appears that chemical disruption of the skin surface with salicylate enhances delivery of antigen by the transcutaneous route.

Table 4. Enhancement of transcutaneous immunization by chemical penetration enhancement: Anti-CT titers in mice that had the skin treated with salicylate (aspirin) before application of the antigen.

		anti-CT IgG (H+L) ELISA units	
Animal #	treatment	prebleed	week 2
741	water		272
742	water		not available
743	water		456
744	water		443
745	water		1395
geometric mean			526
pooled prebleed		7	
756	salicylate/water		2279
757	salicylate/water		4581
758	salicylate/water		4658
759	Salicylate/water		2771
760	Salicylate/water		593
geometric mean			2402
pooled prebleed		36	

Example 5.

To assess the role of physical/mechanical penetration enhancement, an abrasive, in the form of a common emory board, was used to remove a portion of the stratum corneum. BALB/c mice 6 to 8 weeks of age were anesthetized and shaved as described in the "immunization procedure." Twenty four hours later, the backs of the animals were either wiped with a gauze pad saturated in water "water" or brushed 10 times with a medium grain emory board "emory board." and then wiped with a gauze pad saturated in water Approximately five minutes after the water treatment, any excess water was removed and 20 μg of CT (100 μl of a 0.2 mg/ml) solution applied to the back. Removal of excess antigen was conducted as described in the "immunization procedure."

The anti-CT antibody titers were determined using ELISA as described above for "ELISA IgG (H+L)" 3 weeks after a single immunization. The results are shown in Table 5. While CT was clearly immunogenic in both groups, the geometric mean titer in the emory board treated group was 10 fold higher and the titers were more consistent among the latter animals as compared with the "water" animals. Thus it appears that physical disruption of the outer surface of the skin with an emory board enhances delivery of antigen by the transcutaneous route. This can be differentiated from techniques that seek to pierce the skin and deliver antigen through the skin, such as in subcutaneous, intradermal or intramuscular injection.

This simple device could be replaced by other physical disrupting devices to deliver antigens and adjuvants into the epidermis such as microneedles that are of length to disrupt only the stratum corneum or superficial epidermis, devices used for TB tine testing, gas powered guns which do not penetrate the dermis, adhesive tape for tape stripping, or other barrier disruption devices known to disrupt only the stratum corneum or superficial epidermis.

Table 5. Enhancement of transcutaneous immunization by physical penetration enhancement: Anti-CT titers in mice that had the skin treated with an emory board before application of the antigen.

		anti-CT IgG (H+L) ELISA units	
Animal #	treatment	prebleed	week 3
7146	water		1275
7147	water		69
7148	water		7420
7149	water		6025
7150	water		388
geometric mean			1088
pooled prebleed		7	
7151	emory board		6632
7152	emory board		9380
7153	emory board		31482
7154	emory board		11142
7155	emory board		11761
geometric mean			12074
pooled prebleed		9	

Example 6.

Another means of physical/mechanical penetration enhancement was employed using an abrasive pad to remove a portion of the stratum corneum and allow access to the underlying epidermis. BALB/c mice 6 to 8 weeks of age were anesthetized and shaved as described in the "immunization procedure". Twenty four hours later, the backs of the animals were either wiped with a gauze pad saturated in water,"water", or wiped with a gauze pad saturated in water followed by rubbing for 10 seconds with a nylon sponge (buf puf ©) to remove the outermost layers of the stratum corneum, "buf-puf ©". Excess water was removed from the backs of the "water" group and then 20 μg of CT (100 μl of a 0.2 mg/ml solution) was applied to the backs of all animals. Removal of excess antigen was conducted as described in the "immunization procedure."

The anti-CT antibody titers were determined using ELISA as described above for "ELISA IgG (H+L)" 3 weeks after a single immunization. The results are shown in Table 6. While CT was clearly immunogenic in both groups, the geometric mean titer in the buff puff treated group was 2 fold higher and the titers among individual animals were more consistent among the latter animals compared with the "water" animals. Thus it appears that physical disruption of the skin surface with a buff-puf © enhances delivery of antigen by the transcutaneous route.

This simple device could be replaced by other physical penetration devices to deliver antigens and adjuvants into the epidermis such as a needle and tuberculin syringe used for intradermal injection, microneedles that are of length to penetrate only the stratum corneum or superficial dermis, devices used for TB tine testing, abrading patches which have dissolvable crystals such as sucrose or sodium chloride or biodegradable polymers that are impregnated into the patch and rubbed on the skin before securing the patch with antigen either contained in the crystal or in the matrix, gas powered guns, adhesive tape for tape stripping, or other devices known to penetrate only into the epidermis or superficial dermis.

Table 6. Enhancement of transcutaneous immunization by physical penetration enhancement: Anti-CT titers in mice that had the skin treated with an abrasive pad (such as, for example, buf-puf ©) before application of the antigen.

		anti-CT IgG (H+L) ELISA units	
Animal #	treatment	prebleed	week 3
7146	water		1275
7147	water		69
7148	water		7420
7149	water		6025
7150	water		388
geometric mean			1088
pooled prebleed		7	
7166	buf puf©		5376
7167	buf puf ©		2319
7168	buf puf ©		1209
7169	buf puf ©		2871
7170	buf puf ©		2785
geometric mean			2607
pooled prebleed		8	

Example 7.

Transcutaneous immunization with bacterial ADP-ribosylating exotoxins such as CT and LT appear to provide significant 'danger' signals to the immune system stimulating a potent immune response. Such compounds act as adjuvants. It was a surprise to find that simple mixtures of such adjuvants placed on the skin in a manner that hydrates the skin, resulting in potent immune responses. This was described in earlier patents (PCT/US97/21324). However, given that an adjuvant such as CT (86KD) can act as an adjuvant on the skin, it would be expected that other adjuvants, particularly those based on bacterial products or motifs, would be stimulatory when placed on the skin in a manner that hydrates the skin and/or with the use of penetration enhancers.

We used bacterial DNA to confirm that this expectation is correct. BALB/c mice 6 to 8 weeks of age were shaved and anesthetized as described above for the "immunization procedure". On the day of immunization the backs of the mice were wiped with isopropanol to enhance penetration. After the alcohol had evaporated (approximately 5 minutes), 100 µl of phosphate buffered saline (PBS) containing 100 µg of DNA (CpG1 or CpG2) and 100 µg of diphtheria toxoid (DT) was applied to the back for 90 to 120 minutes. Oligonucleotides were synthesized by Oligos Etc with a phosphorothioate backbone to improve stability. Removal of excess antigen was conducted as described in the "immunization procedure." The immunization was repeated 4 and 8 weeks later. Ten weeks after the primary immunization the animals were bled and the anti-DT titers determined using an ELISA as described above for "ELISA IgG (H+L)". The results are shown in Table 7A.

Co-administration of DT and a control DNA sequence (CpG2: *TCCAATGAGCTTCCTGAGTCT*) failed to induce a detectable rise in the anti-DT titers. In contrast, addition of a DNA sequence containing an unmethylated CpG dinucleotide flanked by two 5' purines and two 3' pyrimidines (CpG1 (immunostimulatory DNA): *TCCATGACGTTCCTGACGTT*) resulted in a detectable increase in the serum anti-DT IgG titer in 5 of 5 animals. Thus it appears that bacterial DNA containing appropriate motifs such as CPGs (6KD) can be used as adjuvant to enhance delivery of antigen through the skin for induction of antigen specific antibody responses.

Table 7A. Adjuvant activity of bacterial DNA applied to the skin using penetration enhancement: humoral immune response.

		Anti-DT IgG (H+L) ELISA units	
Animal #	adjuvant/antigen	prebleed	week 10
7261	CpG1/DT		1171
7262	CpG1/DT		22750
7263	CpG1/DT		4124
7264	CpG1/DT		126
7265	CpG1/DT		115
Geometric mean pooled prebleed		6	1096
7266	CpG2/DT		19
7267	CpG2/DT		12
7268	CpG2/DT		5
7269	CpG2/DT		5
7270	CpG2/DT		11
geometric mean pooled prebleed		5	9

The transcutaneous effect of transcutaneous immunization can also be detected by T-cell proliferation. BALB/c mice 6 to 8 weeks of age were shaved and anesthetized as described above for the "immunization procedure". On the day of immunization the backs of the mice were wiped with isopropanol. After the alcohol had evaporated (approximately 5 minutes), 100 µl of phosphate buffered saline (PBS) containing 100 µg of DNA (CpG1 or CpG2) and 100 µg of diphtheria toxoid (DT) was applied to the back for 90 to 120 minutes. Oligonucleotides were synthesized by Oligos Etc with a phosphorothioate backbone to improve stability. Removal of excess antigen was conducted as described in the "immunization procedure." The immunization was repeated 4 and 8 weeks later. Twelve weeks after the primary immunization draining (inguinal) LNs were removed and pooled from five immunized animals. The capacity

to proliferate in response to media or antigen (DT) was assessed in a standard 4 day proliferation assay using 3-H incorporation as a readout. The results are shown in Table 7B. Co-administration of DT and a DNA sequence containing an unmethylated CpG dinucleotide flanked by two 5' purines and two 3' pyrimidines (CpG1 (immunostimulatory DNA): *TCCATGACGTTCCTGACGTT*) resulted in a detectable increase in the antigen specific proliferative response. Thus it appears that bacterial DNA containing appropriate motifs can be used as adjuvant to enhance delivery of antigen through the skin for induction of proliferative responses.

Table 7B. Adjuvant effect of bacterial DNA applied to the skin: LN cell proliferation

	proliferation (cpm) 3-H incorporation in vitro to antigens	
antigens applied in vivo	media	DT
normal LN	339	544
CpG1/DT	1865	5741

Example 8.

Transcutaneous immunization with bacterial ADP-ribosylating exotoxins such as CT and LT appear to provide significant 'danger' signals to the immune system stimulating a potent immune response. Such compounds act as adjuvants. It was a surprise to find that simple mixtures of such adjuvants placed on the skin in a manner that hydrates the skin, resulting in potent immune responses. This was described in earlier patents (PCT/US97/21324). However, given that an adjuvant such as CT can act as an adjuvant on the skin, it would be expected that other adjuvants, would be stimulatory when placed on the skin in a manner that hydrates the skin. Genetically altered toxins were used to confirm this expectation. BALB/c mice 6 to 8 weeks of age were anesthetized, shaved, and immunized as described in the "immunization procedure". The animals were boosted 3 and 5 weeks after the primary immunization and serum collected 2 weeks after the final immunization. The adjuvants used were the genetically altered toxins; LTK63, an enzymatically inactive LT derivative, and LTR72, an LT derivative which retains 0.6% of the enzymatic activity. Diphtheria toxoid (DT) 100 μg was used as antigen.

Anti-DT antibody titers were determined using ELISA as described above for "ELISA IgG (H+L)". The results are shown in Table 8. Anti-DT titers were clearly elevated in serum from animals immunized with either LTR63 or LTR72 and DT when compared with titers in serum collected prior to immunization (prebleed). Thus it appears that genetically detoxified mutants of heat labile enterotoxin (LT) can be used as adjuvants on the skin.

Table 8. Use of genetically altered toxins, LTK63 and LTR72, as adjuvants on the skin.

	anti-DT IgG (H+L) ELISA units		
Animal #	adjuvant / antigen	prebleed	week 7
653	LTK63 / DT		20228
654	LTK63 / DT		not available
655	LTK63 / DT		342
656	LTK63 / DT		2445
657	LTK63 / DT		< 100
geometric mean			1140
pooled prebleed		<100	
663	LTR72 / DT		12185
664	LTR72 / DT		10917
665	LTR72 / DT		151
666	LTR72 / DT		2057
667	LTR72 / DT		50923
geometric mean			4620
pooled prebleed		<100	

Example 9.

Another class of compounds, cytobines which are known to act as adjuvants illustrate the principle that adjuvants in general could be expected to act in a fashion similar to cholera toxin. TNFα is also known to be a Langerhan cell activating compound.

BALB/c mice 6 to 8 weeks of age were shaved and anesthetized as described above for the "immunization procedure". On the day of immunization the backs of the mice were wiped with isopropanol. After the alcohol had evaporated (approximately 5 minutes), 100 µl of phosphate buffered saline (PBS) containing 0.83 µg TNF-alpha (recombinant mouse TNF-alpha, Endogen), IL-2 (1 µg recombinant mouse IL-2 (Sigma)) or mock adjuvant (CpG2) was applied to the skin on the back with 100 µg of diphtheria toxoid (DT) for 90 to 120 minutes. Oligonucleotides were synthesized by Oligos Etc with a phosphorothioate backbone to improve stability Removal of excess antigen was conducted as described in the "immunization procedure." The immunization was repeated 4 and 8 weeks later. Ten weeks after the primary immunization the animals were bled and the anti-DT titers determined using an ELISA as described above for "ELISA IgG (H+L)". The results are shown in Table 9.

Co-administration of DT and a mock adjuvant (CpG2) failed to induce a detectable rise in the anti-DT titers. In contrast, topical application of TNF-alpha (0.8 µg) resulted in a detectable increase in the serum anti-DT IgG titer in 3 of 5 animals when compared with either anti-DT titers in the mock adjuvant treated mice or sera collected prior to immunization (prebleed). Similarly, topical application of IL-2 (1 µg) resulted in a detectable increase in the serum anti-DT IgG titer in 4 of 5 animals when compared with either anti-DT titers in the mock adjuvant treated mice or sera collected prior to immunization (prebleed). Thus it appears that the cytokines such as IL-2 and TNF-alpha can be used as an adjuvant on the skin and that langerhans cell activating compounds can be used for transcutaneous immunization.

Table 9. Adjuvant activity of the cytokine TNF-alpha applied to the skin.

Animal #	adjuvant/antigen	Anti-DT IgG (H+L) ELISA units prebleed	week 10
7326	TNF-alpha/DT		1808
7327	TNF-alpha/DT		830
7328	TNF-alpha/DT		7
7329	TNF-alpha/DT		1477
7330	TNF-alpha/DT		7
geometric mean pooled prebleed		1	159
7331	IL-2/DT		13
7332	IL-2/DT		111
7333	IL-2/DT		345
7334	IL-2/DT		49
7335	IL-2/DT		35
geometric mean pooled prebleed		2	61
7266	CpG2/DT		19
7267	CpG2/DT		12
7268	CpG2/DT		5
7269	CpG2/DT		5
7270	CpG2/DT		11
geometric mean pooled prebleed		5	9

Example 10.

The B-subunit of cholera toxin is another class of adjuvants lack the A-subunit and therefore ADP-ribsyltransferase activity of CT. As such CTB represents an adjuvant that is unique and may be useful as it is not toxic when ingested.

C57BL/6 mice 6 to 8 weeks of age were anesthetized and shaved as described in the "immunization procedure". On the day of immunization the backs of the mice were wiped with isopropanol. After the alcohol had evaporated (approximately 5 minutes), 100 µl of phosphate buffered saline (PBS) containing 100 µg of purified cholera toxin B subunit (CTB) and/or 100 µg of diphtheria toxoid (DT) was applied to the back for 90 to 120 minutes. Removal of excess antigen was conducted as described in the "immunization procedure." The immunization was repeated 4 and 8 weeks later. Ten weeks after the primary immunization the animals were bled and the anti-DT titers determined using an ELISA as described above for "ELISA IgG (H+L)". The results are shown in Table 10.

Anti-DT titers were clearly elevated in serum from animals immunized with CTB and DT when compared with titers in serum from animals treated with DT alone or those in prebleed serum samples as shown in Table 10. Thus it appears that purified CTB can be used as an adjuvant on the skin.

Table 10. Use of purified cholera toxin B subunit from *V. cholerae* as an adjuvant on the skin.

	Anti-DT IgG (H+L) ELISA units		
Animal #	adjuvant / antigen	prebleed	week 10
51	DT		11
52	DT		7
53	DT		4
54	DT		8
55	DT		7
geometric mean			7
pooled prebleed		4	
81	CTB/DT		14880
82	CTB/DT		371
83	CTB/DT		14810
84	CTB/DT		108
85	CTB/DT		27
geometric mean			751
pooled prebleed		5	

Example 11.

Adjuvants that are structurally different are likely to exert their enhancement by different effects. Adjuvants that induce their effects by different mechanisms may have either additive or synergistic effects on enhancing the immune response. We found that the use of two adjuvants simultaneously augmented the response to transcutaneous immunization compared to the individual adjuvants alone.

BALB/c mice 6 to 8 weeks of age were shaved and anesthetized as described above for the "immunization procedure". On the day of immunization the backs of the mice were wiped with isopropanol. After the alcohol had evaporated (approximately 5 minutes), 100 µl of phosphate buffered saline (PBS) containing 100 µg of immunostimulatory DNA (CpG1) and/or cholera toxin (CT) 100 µg was applied to the back with 100 µg of a soluble leishmanial antigen extract (SLA) for 90 to 120 minutes. SLA is an antigen extract prepared at Walter Reed Army Institute of Research by centrifugal isolation of the soluble proteins in a sonicate of *Leishmania major* promastigotes) extract for 90 to 120 minutes. Removal of excess antigen was conducted as described in the "immunization procedure." The immunization was repeated 4 and 8 weeks later. Twelve weeks after the primary immunization draining (inguinal) LNs were removed and pooled from two immunized animals. The capacity to proliferate in response to media or antigen (SLA) was assessed in a standard 4 day proliferation assay using 3-H incorporation as a readout. The results are shown in Table 11.

Co-administration of SLA and CpG1 (immunostimulatory DNA containing an unmethylated CpG dinucleotide flanked by two 5' purines and two 3' pyrimidines - *TCCATGACGTTCCTGACGTT*) or CT resulted in a detectable increase in the antigen specific proliferative response. However, the antigen (SLA) specific proliferative response was approximately 20 times higher in lymph node cell cultures from animals exposed simultaneously to both CpG1 and CT as compared to cultures derived from animals exposed to either adjuvant alone. Thus it appears that bacterial DNA containing appropriate motifs synergizes with ADP ribosylating exotoxins such as CT as adjuvants on the skin to induce higher immune responses that to either adjuvant alone.

Table 11. Synergy between immunostimulatory DNA and ADP ribosylating exotoxin (CT) as adjuvants when applied to the skin

	proliferation (cpm) 3-H incorporation in vitro to antigens	
substances applied in vivo	media	SLA
normal LN	180	219
SLA	200	159
SLA/CpG1	1030	2804
SLA/CT	232	2542
SLA/CpG1/CT	2232	47122

Example 12.

Transcutaneous immunization induces potent immune responses when used as a method of delivery alone. We also have found that transcutaneous immunization can be used in sequence with other routes of delivery to stimulate an immune response.

BALB/c mice 6 to 8 weeks of age On day 0 both groups of animals received a 50 µl intramuscular (im.) injection of DT (5 µg) mixed with alum (Rehydrogel - 25 µg in NaCl) into the hind thigh. Eight and 16 weeks later mice in the im/tc/tc group were shaved, anesthetized and immunized by the transcutaneous route as described above for the "immunization procedure" using 100 µg cholera toxin as adjuvant and 100 µg diphtheria toxoid as antigen. The immunization solution was applied to the back for 90 to 120 minutes. Removal of excess antigen was conducted as described in the "immunization procedure." Twenty two weeks after the primary immunization the animals were bled and the anti-DT titers determined using an ELISA as described above for "ELISA IgG (H+L)". The results are shown in Table 12.

A single im. injection of 5 µg of DT induced a detectable rise in the serum anti-DT titers as compared with titers in sera collected from the same animals prior to immunization (prebleed). Boosting of the im. primed animals using the transcutaneous immunzation method resulted in an 60 fold rise in the geometric mean titer and clearly all transcutaneously boosted animals had higher anti-DT titers that those observed in the im. primed group. Thus transcutaneous immunization can be

used to boost antigen specific titers in mice in which the primary immunization with the antigen was by the i.m. route. We have also found that im primed animals can be boosted by transcutaneous immunization (TCI). Various combinations of TCI priming or boosting with other routes and schedules can be visualized including oral, buccal, nasal, rectal, vaginal, intradermal, by gun or other means of delivery. Additionally, antigens may differ in route and composition including protein alternating with glycoprotein, subunit with holotoxin, DNA priming followed by protein, nucleic acid by im followed by nucleic acid by TCI. Transcutaneous immunization may be used to boost children primed in infancy or adults primed in childhood. The ease of delivery may enhance the efficacy vaccines such as the influenza vaccines by allowing multiple boosts using a patch.

Table 12. Boosting of im. primed animals using the transcutaneous immunization method

			Anti-DT IgG (H+L) ELISA units	
Animal #	adjuvant/antigen	Route of administration	prebleed	week 22
8563	DT	im.		54227
8564	DT	im.		11833
8565	DT	im.		106970
8566	DT	im.		10830
8567	DT	im.		4003
geometric mean				19711
pooled prebleed			20	
8568	DT/ct+dt/ct+dt	im./tc./tc.		628838
8569	DT/ct+dt/ct+dt	im./tc./tc.		2035507
8570	DT/ct+dt/ct+dt	im./tc./tc.		1164425
8571	DT/ct+dt/ct+dt	im./tc./tc.		not available
8572	DT/ct+dt/ct+dt	im./tc./tc.		1263138
geometric mean				1171368
pooled prebleed			10	
8558	DT /DT/DT	im./im./im.		not available
8559	DT /DT/DT	im./im./im.		542669
8560	DT /DT/DT	im./im./im.		770150
8561	DT /DT/DT	im./im./im.		545894
8562	DT /DT/DT	im./im./im.		671898
geometric mean				625721
pooled prebleed			15	

Example 13.

Because TCI appears to stimulate the immune system in such a potent fashion, it is possible that an adjuvant place on the skin at one site may act as an adjuvant for an antigen placed at another site. BALB/c mice 6 to 8 weeks of age were anesthetized and shaved as described in the "immunization procedure". Animals were not ear tagged but kept in cages labeled A, C or G. On the day of immunization the dorsal surface of the mouse ear was treated by gently rubbing the outer skin surface with a cotton-tipped applicator containing 70% isopropanol. After 5 minutes the excess water was blotted from water-treated ears and adjuvant (CT 50 μg) and/or antigen (100 μg of bovine serum albumin (BSA) was applied to the left or right ear surface (described in table) in 50 μl phosphate buffered saline. At two and a half hours, the ears were rinsed and blotted dry twice. Mice were boosted in a similar fashion four and eight weeks later. Twelve weeks after the primary immunization the animals were bled and the anti-BSA titers determined using an ELISA as described above for "ELISA IgG (H+L)". The results are shown in Table 13.

Application of BSA alone to the skin was poorly immunogenic with only 1 of 5 animals developing an ELISA titer above 100 EU. In contrast, 9 of 9 animals receiving CT and BSA on the skin developed titers above 100 EU. Of the animals receiving antigen and adjuvant, the mice given the materials at the same site (left ear) developed higher (10 fold) anti-BSA titers than animals receiving antigen and adjuvant in separate (left and right) ears. However, animals receiving antigen on one ear and adjuvant on another ear developed an anti-BSA immune response that was approximately 30 times higher that animals given BSA alone. Thus, antigen and adjuvant may be topically applied during TCI at different sites to elicit a humoral immune response. This immunostimulation may be expected to occur with antigen delivered by other routes and schedules can be visualized including oral, buccal, nasal, rectal, vaginal, intradermal, by gun or other means of delivery. Additionally, adjuvants may be used with nucleic acid immunization to enhance the response. Such a delivery may not need to be simultaneous to enhance the immune response. For example, an im injection of plasmid DNA may be followed later by transcutaneous administration of adjuvant. Immunostimulation by CT, LT, TNFα, CpGs or similar

adjuvants is a surprising result because it has been thought that molecules 500 daltons could not pass through the skin.

Table 13. Delivery of antigen and adjuvant at the same or distal sites on the skin with penetration enhancement.

	Anti-BSA IgG (H+L) ELISA units		
Animal #	adjuvant / antigen	prebleed	week 12
group G	BSA left ear		240
group G	BSA left ear		99
group G	BSA left ear		40
group G	BSA left ear		not available
group G	BSA left ear		15
Geometric mean pooled prebleed		6	61
group C	CT/BSA left ear		16418
group C	CT/BSA left ear		24357
group C	CT/BSA left ear		13949
group C	CT/BSA left ear		70622
group C	CT/BSA left ear		not available
Geometric mean pooled prebleed		3	25053
group A	CT left/BSA right ear		106
group A	CT left/BSA right ear		23806
group A	CT left/BSA right ear		1038
group A	CT left/BSA right ear		1163
group A	CT left/BSA right ear		8696
Geometric mean pooled prebleed		15	1939

Example 14. Transcutaneous Immunization in Humans

The invention can be practiced using a suitable vehicle or carrier. For example a patch can be used as such a vehicle and can be treated with the formulation of the invention or can be used to cover the area of the skin which has been treated with the formulation of the invention. A suitable patch can be fabricated from, for example, cotton, nylon, rayon, polyester or combinations thereof. Such patches can be provided with an adhesive or non-adhesive backing. Patches with a non-adhesive backing can be secured to the animal by non-adhesive means, such as, for example,wrapping. Suitable backing can be fabricated from materials such as, for example, silicon, acrylate or rubber. Other vehicles or carriers which can be used include those listed above; such as for example, powders, oils, water, cream and the like. To evaluate the potential for TCI in humans, a Phase I trial was conducted using LT to attempt to induce serum anti-LT antibodies. Six volunteers received a dose of 500 ug of LT, a dose similar to oral adjuvant doses used for a cholera vaccine (1 mg CTB). LT was produced under GMP conditions at the Swiss Serum and Vaccine Institute (Berne, Switzerland) and was provided by Oravax Inc., Cambridge, MA. The volunteers received 500 ug of LT mixed in 500 ul of sterile saline which was absorbed on a $2in^2$ cotton gauze pad with polyvinyl backing and covered by a 4x4 in^2 Tegaderm™ dressing. The immunization was conducted by placing the patch on unmanipulated skin for 6 hours after which the site was thoroughly rinsed with 500 ml of sterile saline. Individuals were similarly reimmunized after 12 weeks. Volunteers were examined on days 1, 2, 3 and 7 for signs of inflammation at the site of immunization and interviewed for symptoms related to the vaccination. The immunization was conducted by placing the patch on unmanipulated skin for 6 hours after which the patch was removed and the site was thoroughly rinsed with saline. Individuals were reimmunized after 12 weeks. No adverse reactions were seen, either systemically or at the site of immunization after the first or second immunization. Anti-LT IgG titers were determined as previously described. Results are reported in ELISA units (EU) which are defined as the inverse dilution of sample that yields an OD of 1.0. Anti-LT IgA was determined in the same manner as anti-LT IgG using goat anti-human IgA(α)-HRP (Kirkegaard and Perry, Gaithersburg, MD) against a standard IgA curve made using human IgA (ICN). As shown in Table 14, 6 of 6 individuals responded

by producing a rise in serum anti-LT IgG or IgA antibodies, defined as a four-fold increase in antibody titers. The mean fold rise in anti-LT IgG was 10.2 and the mean fold rise in serum anti-LT IgA was 7.2. Biopsies of the immunization site and contralateral arm showed no signs of inflammation of the skin. These results confirm that TCI can be conducted in humans without skin irritation or inflammation.

Suitable patch materials have been previously described. In general the patch may consist of, for example, a pressure sensitive dressing, a matrix for or absorbant layer to carry the vaccines and adjuvant, a vaccine impermeable backing and a release liner. This and other suitable patch examples are described in U.S. Patent Nos. 4,915,950 and 3,734,097.

Patches can be fabricated to include woven and non-woven matrices of materials to include, for example, polyester/cellulose, polypropylene, polyester, polyester/rayon and the like.

Examples of non-woven patch matrices can include:

BBA Nonwovens

a. Grade# 1313290, wet laid non-woven, composition = polyester/cellulose, weight (gsy) = 35.4, weight (gsm) = 42.3, thickness (mils) = 7.9, tensile MD = 3.4, tensile CD = 2.4.

b. Grade# 2006086, thermal bond non-woven, composition = polypropylene, weight (gsy) = 16.0, weight (gsm) = 19.1, thickness (mils) = 10.2, tensile MD = 3.3, tensile CD = 0.7.

c. Grade# 149146, thermal bond non-woven, composition = polyester, weight (gsy) = 25.6, weight (gsm) = 30.6, thickness (mils) = 6.5, tensile MD = 5.3, tensile CD = 0.9.

d. Grade# 149020, thermal bond non-woven, composition = polyester/rayon, weight (gsy) = 30.5, weight (gsm) = 36.4, thickness (mils) = 13.2, tensile MD = 5.5, tensile CD = 1.1.

e. Grade# 140-027, hydroentangled nonwoven, composition = polyester/rayon, weight (gsy) = 28.0, weight (gsm) = 33.5, thickness (mils) = 22.4,

tensile MD = 10.4, tensile CD = 3.8.

Pressure sensitive adhesive that can be used in the present invention include, for example adhesive based on acrylate, silicone, rubber and the like.

Table 14 Mean fold rise in human anti-LT IgG and IgA

Volunteer #	4 week IgG	12 week IgG	16 week IgG
13	15.2	9.5	12.5
14	1.4	1.6	1.7
15	11.7	15.0	12.9
16	1.3	0.7	16.0
17	12.5	51.9	58.6
18	1.3	2.1	4.3
Mean rise IgG	4.2	5.0	10.2
Volunteer #	4 week IgA	12 week IgA	16 week IgA
13	7.2	4.1	10.1
14	4.9	4.3	4.3
15	4.9	5.7	4.5
16	1.4	1.3	7.0
17	15.3	29.4	28.1
18	1.3	1.5	3.5
Mean rise IgA	4.1	4.2	7.2

Example 15.

LT has been shown to be effective for immunizing humans by the transcutaneous route. We also have found that LT acts as an adjuvant for TCI. C57BL/6 mice 6 to 8 weeks of age were anesthetized and shaved as described in the "immunization procedure". On the day of immunization the backs of the mice were wiped with isopropanol. After the alcohol had evaporated (approximately 5 minutes), 100 µl of phosphate buffered saline (PBS) containing 100 µg of heat labile enterotoxin (LT) and/or 100 µg of diphtheria toxoid (DT) was applied to the back for 90 to 120 minutes. Removal of excess antigen was conducted as described in the "immunization procedure." The immunization was repeated 4 and 8 weeks later. Ten weeks after the primary immunization the animals were bled and the anti-DT titers determined using

an ELISA as described above for "ELISA IgG (H+L)". The results are shown in Table 15.

Anti-DT titers were clearly elevated in serum from animals immunized with LT and DT when compared with titers in serum from animals treated with DT alone or those in prebleed serum samples. Thus it appears that heat labile enterotoxin (LT) can be used as an adjuvant on the skin.

Table 15. Use of heat labile enterotoxin (LT) from *E. coli* as an adjuvant on the skin.

	Anti-DT IgG (H+L) ELISA units		
Animal #	adjuvant / antigen	prebleed	week 10
51	DT		11
52	DT		7
53	DT		4
54	DT		8
55	DT		7
geometric mean pooled prebleed		4	7
71	LT/DT		7126
72	LT/DT		46909
73	LT/DT		669
74	LT/DT		8480
75	LT/DT		1598
geometric mean pooled prebleed		5	4970

Example 16.

To assess the role of physical/mechanical penetration enhancement, the superficial layers of the stratum corneum were removed by tape stipping. Tape stripping is an intervention well known in the art to remove the outer layer of the stratum corneum. C57BL/6 mice 6 to 8 weeks of age were anesthetized and shaved as described in the "immunization procedure." Twenty four hours later, CT (25 μg) was applied to the backs of the mice in 50 μl of phosphate buffered saline; "none" intervention group. Alternatively, the skin on the backs of a second group of animals was subjected to mild tape stripping; "tape stripping" intervention group. The tape stripping procedure was accomplished by applying cellophane scotch-tape to the backs, allowing bonding to the skin surface for 3 minutes, followed by gentle removing of the tape. The bonding/removalsteps were repeated 3 times. CT (25 μg) was then applied to the backs of the mice in 50 μl of phosphate buffered saline. Antigen remained on the backs for approximately 1.5 hrs at which time removal of excess antigen was conducted as described in the "immunization procedure."

The anti-CT antibody titers were determined using ELISA as described above for "ELISA IgG (H+L)" using sera collected 11 days after the primary immunization. The results are shown in Table 16. CT was immunogenic in both groups as compared to sera collected from the same animals prior to immunization (prebleed). However, the geometric mean titer in the tape stripped group was 100 fold higher and the titers were more consistent among the latter animals as compared with the "none" animals. Thus it appears that physical disruption of the skin surface using tape stripping enhances delivery of antigen by the transcutaneous route.

This simple device could be replaced by other physical penetration devices to deliver antigens and adjuvants into the epidermis such as a needle and tuberculin syringe used for intradermal injection, microneedles that are of length to penetrate only the stratum corneum or superficial dermis, devices used for TB tine testing, gas powered guns, adhesive tape for tape stripping, or other devices known to penetrate only into the epidermis or superficial dermis. Tape stripping devices could be used in conjunction with other penetration enhancers. Tape stripping devices may be used in conjunction with a marker to delineate the site for patch placement, and may be dispersed in a roll or in individual units.

Table 16. Enhancement of transcutaneous immunization by physical penetration enhancement: Anti-CT titers in mice that had the skin stripped using cellophane tape before application of the antigen.

		anti-CT IgG (H+L) Elisa units	
Animal #	intervention	prebleed	day 11
976	none		155
977	none		4
978	none		4
979	none		31
980	none		23
geomean prebleed		2	16
986	tape strip		10702
987	tape strip		1285
988	tape strip		5832
989	tape strip		997
990	tape strip		782
geomean prebleed		3	2990

Example 17.

Nucleic acids such as plasmid DNA or RNA can be used to induce and immune response and are well known in the art. The use of Nucleic acids in transcutaneous immunization was described in previous patents (PCT/US97/21324). The use of nucleic acids (also known as genetic immunization) on the skin with penetration enhancement techniques is illustrated in the following example.

C57BL/6 mice 6 to 8 weeks of age were anesthetized and shaved as described in the "immunization procedure". For the "NP DNA" group the mice were wiped with isopropanol, after the alcohol had evaporated (approximately 10 minutes), the backs were hydrated with water using a saturated gauze pad. Approximately 10 minutes later the any excess water was blotted off and a 100 μg of a DNA plasmid (pCMV-NP) encoding for influenza nucloprotein was applied to the back in 100 μl of

saline. A second group of NP-DNA mice were subjected to the same immunization protocol except that their backs were tape stripped 3x prior to alcohol swabbing; "NP DNA – tape stripping". The tape stripping procedure was accomplished by applying cellophane scotch-tape to the backs, allowing bonding to the skin surface for 5 minutes, followed by gentle removing of the tape. A third group of mice was engaged in the tape stripping / immunization protocol described and 100 μg of the adjuvant heat labile enterotoxin (LT) was included in the immunization solution. Sixteen days after the primary immunization the animals were bled and the anti-influenza NP titers determined using an ELISA as described above for "ELISA IgG (H+L)". The results are shown in Table 17.

Anti-influenza titers were determined using a split virus antigen (Fluzone) preparation to coat the ELISA plates. ELISA titers were determined in 5 individual animals and the mean optical density reading for each group is shown. All three immunization groups developed anti-influenza titers as compared with titers in serum collected from the same animals prior to immunization (prebleed). As compared with the NP DNA alone group, tape stripping prior to immunization enhanced the anti-Influenza titer in all three serum dilutions tested (1:100, 1:200, 1:400) and addition of an adjuvant (LT) further enhanced this response. Thus, DNA can be used on the skin to induce immune responses to vaccine antigens and its effectiveness can be enhanced by the addition of adjuvants and penetration enhancement such as tape stripping.

Table 17. Immuogenicity of DNA applied as antigen on the skin using alcohol penetration enhancement.

		Anti-INF IgG optical density (405 nm)			
		prebleed	day 16 post immunization		
Antigen/adjuvant	intervention	1:100	1:100	1:200	1:400
NP DNA	none	0.21	0.47	0.20	0.07
NP DNA	tape stripping	0.39	0.64	0.28	0.13
NP DNA / LT	tape stripping	0.39	0.87	0.38	0.13

Example 18.

Transcutaneous immunization (TCI), because of the ease of delivery, allows the application to be given over different draining lymph nodes. This may have an additional advantage in that it enhances the immune response. Rabbits were anesthetized, shaved, and immunized as described above. Animals were immunized with 100 μg cholera toxin (CT) and 100 μg of influenza hemagglutinin (HA) at one site or two sites on the back. HA and CT were applied at 0, 3 and 5 weeks. Seven weeks after the primary immunization, the animals were bled and the anti-HA titers determined using an ELISA as described above for "ELISA IgG (H+L)". The results are shown in Table 18.

Anti-HA titers were elevated in serum from 10 of 10 animals immunized with CT and HA when compared with titers in serum from the same animals prior to immunization (prebleed). The geometric mean titer in the two site group was 3 fold higher than that in the one site group suggesting that antigen delivery at multiple sites may be used to enhance TCI. Thus, antigens can be delivered by TCI either at a single or multiple sites on the skin.

Table 18. Transcutaneous delivery of antigen in a single or multiple sites.

Animal	antigen/adjuvant	anti-HA IgG (ELISA units)		
		prebleed	7 weeks	geomean
1	CT/HA one site	< 25	1142	2596
2	CT/HA one site	< 25	9617	
3	CT/HA one site	< 25	2523	
4	CT/HA one site	< 25	2275	
5	CT/HA one site	< 25	1869	
6	CT/HA two sites	< 25	10348	8403
7	CT/HA two sites	< 25	18453	
8	CT/HA two sites	< 25	9778	
9	CT/HA two sites	< 25	15985	
10	CT/HA two sites	< 25	1404	

Example 19

The variety of antigens which can be delivered by TCI is further illustrated by the use of a polysaccharide conjugate vaccine to induce anti-polysaccharide antibodies. BALB/c mice 6 to 8 weeks of age were anesthetized, shaved, and immunized as described in the "immunization procedure". Mice were immunized with cholera toxin (CT) and *Haemophilus influenzae B* polysaccharide (Hib-PS) at 0, 3 and 5 weeks. Seven weeks after the primary immunization, the animals were bled and the anti-Hib-PS titers determined using an ELISA as described above for "ELISA IgG (H+L)". The results are shown in Table 19.

Anti-Hib-PS titers were elevated in serum from 4 of 10 animals immunized with CT and Hib-PS when compared with titers in serum from the same animals prior to immunization (prebleed). Thus TCI can be used to induce anti-polysaccharide antigens through the skin. This is a common human use vaccine antigen and represents and important strategy for immunization.

Table 19. Delivery of a conjugated polysaccharide by transcutaneous immunization

ear tag #	antigen/adjuvant	anti-Hib PS IgG (μg/ml)	
		prebleed	7 weeks
1	CT/Hib-PS (100μg/100μg)	< 0.20	< 0.20
2	CT/Hib-PS (100μg/100μg)	< 0.20	< 0.20
3	CT/Hib-PS (100μg/100μg)	< 0.20	1.68
4	CT/Hib-PS (100μg/100μg)	< 0.20	< 0.20
5	CT/Hib-PS (100μg/100μg)	< 0.20	1.86
6	CT/Hib-PS (100μg/25μg)	< 0.20	1.04
7	CT/Hib-PS (100μg/25μg)	< 0.20	< 0.20
8	CT/Hib-PS (100μg/25μg)	< 0.20	< 0.20
9	CT/Hib-PS (100μg/25μg)	< 0.20	6.30
10	CT/Hib-PS (100μg/25μg)	< 0.20	< 0.20

Example 20

Transcutaneous immunization of mice with human use vaccine antigens

CT has been shown to act as adjuvant for transcutaneous immunization with single toxoids and BSA. We transcutaneously immunized mice with a variety of human-use vaccine antigens, including a multivalent toxoid vaccine (tetanus and diphtheria toxoids), a yeast expressed recombinant protein(HIV p55 gag) and whole killed rabies viruses using CT as an adjuvant as shown in Table 20. BALB/c mice (n=5) were immunized and boosted twice as previously described (Glenn, G.M., Scharton-Kersten, T., Vassell, R., Matyas, G. & Alving. C.R. Transcutaneous immunization using bacterial ADP-ribosylating exotoxins as antigens and adjuvants. *Infect. Immun.* (in the press). Immunizing doses included 100/50/50 ug CT/TT/DT via TCI versus 3/1/1 for alum/TT/DT IM; 100/100 ug for LT+DT versus 100 ug of DT alone. 100/100 ug for CT /p55 via TCI versus 100 ug p55 alone. Mice immunized with 17 IE of killed rabies virus (n=10) had been primed intramuscularly 2x and then boosted transcutaneously (17 IE) after light alcohol swabbing of the skin and compared to 3x IM rabies immunization. Antibody levels against DT, TT, p55 and rabies were determined using ELISA as previously described (Grassi, M., Wandler, A. & Peterhans, E. Enzyme-linked immunoabsorbant assay for determination of antibodies to the envelope glycoprotein of rabies virus. *J.Clin.Microbiol.* 27, 899-902 (1989). Miyamura, K., Tajiri, E., Ito, A., Murata, R. & Kono, R. Micro cell culture method for determination of diphtheria toxin and antitoxin titres using VERO cells. II. Comparison with the rabbit skin method and practical application for seroepidemiological studies. *J. Biol. Stand.* 2, 203-209 (1974)). TCI resulted in similar increases in the antibody responses to TT and DT and the anti-DT neutralization titers were comparable to that elicited by intramuscular immunization (IM). These data show that TCI may be used to inudce immune response of comparable magnitude as those induced by existing immunization practices. TCI boosting of IM primed animals also resulted in a significant rise in anti-rabies titers in all 10 animals tested (0.53 to 1.03 IU, $p<0.02$, Student's t-test). Antibodies to the antigens DT and p55 administered without adjuvants were very low or undetectable, consistent with our previous observations that antigens are only weakly immunogenic when applied without adjuvant. LT also acted as adjuvant (Table 20) in a fashion

similar to previous studies using CT (1,2). Although the immunizations were not optimized as compared to intramuscular delivery, these antigen-specific responses confirm that TCI may be used for a variety of human-use vaccines from a variety of sources an a variety of sizes and that LT can act as an adjuvant for coadministered vaccine antigens.

Table 20.

Murine antibody responses to human-use vaccine antigens administered by TCI

Immunizing Antigen(s)for TCI	Antibody specificity	TCI (ELISA Units)	IM/alum (ELISA Units)
CT+TT+DT	Anti-DT	135,792 (86,552-146-759)	85,493 (24,675-238,904)
CT+TT+DT	Anti-TT	30,051 (13,863-53,174)	94,544 (74,928-113,408)
CT+TT+DT	Diphtheria toxin neutralization	404 (22-2816)	1,226 (352-11,264)
LT+DT	Anti-DT	4976 (669-46,909)	ND
CT+HIV p55 gag	Anti-p55	10,630 (1063-52,597)	ND
CT+Killed Rabies Virus	Anti-G protein	1.03 (IU/ml) (0.31-2.77)	7.54 (IU/ml) (3.31-17.47)

ND=not done. ELISA units (EU) shown as geometric mean and range in brackets.

Example 21

Langerhans cell activation

In two subjects, the site of immunization and the contralateral unimmunized arm were biopsied, one at 24 hours post-immunization and one at 48 hours after the second immunization. Hematoxylin and eosin (H&E) staining of the specimens confirmed the clinical findings suggesting that no inflammation was seen after the immunization (Fig 1 A,B). Although routine histologic sections were unremarkable, LCs visualized using anti-CD1a staining of specimens from the site of immunization demonstrated greatly enlarged cell bodies but otherwise normal numbers of cells when compared to the control biopsies from the opposite arm, both at 24 and 48 hours (Fig. 1 C,D,E,F). Similar findings were seen using anti-HLA-DR and anti-S-100 to visualize LCs (not shown). LC morphology in the TCI immunized skin was similar in appearance to tonsilar crypt LCs that are thought to be chronically activated by lipopolysaccharides from the flora of the mouth (Noble).

Because of the limited size and number of human skin biopsy specimens examined, complementary murine studies were performed. LC activation in murine systems using contact sensitizers, LPS, and proinflammatory cytokines is characterized by both changes in morphology (Aiba) and through elevations in surface marker expression (Jakob and Udey). Mouse ear skin is frequently used for studies of LC activation and has also been shown to be an excellent site for transcutaneous immunization (Scharton-Kersten). Epidermal sheets were prepared 24 hours after application of CT to the ear and stained for MHC class II, an LC-restricted marker in murine skin. In comparison to the PBS treated ears, Fig. 2A, LC in CT treated ears exhibited marked changes in LC morphology with loss of dendritic processes, enlarged cell bodies, and intense staining of the cells - features of LC activation (Aiba) (Fig. 2 B,C). The LC-activating potential of CT was confirmed using flow cytometry. LC from CT-treated skin expressed increased levels of MHC Class II antigens and CD 86 (B7-2) and decreased levels of E-cadherin, consistent with LC activation described elsewhere (Pierre, Aiba, Jakob).

Immunization Procedure which may be used for Example 22.

BALB/c mice may be shaved with a #40 clipper. This shaving could be done without any signs of trauma to the skin. The shaving could be done from the mid-thorax to just below the nape of the neck. The mice can then be allowed to rest for 24 hours. Prior to this the mice could be ear-tagged for identification, and pre-bled to obtain a sample of pre-immune serum. Mice could also be transcutaneously immunized without shaving by applying 5-500 µl of immunizing solution to each ear. The mice could be immunized in the following way. Mice could be anesthetized with 0.03-0.06 ml of a 20 mg/ml solution of xylazine and 0.5 ml of 100 mg/ml ketamine and immobilized by this dose of anesthesia for approximately 1-3 hours. The mice could be placed ventral side down on a warming blanket.

The immunizing solution and penetration enhancement compound (or technique) could be placed on the dorsal shaved skin of a mouse in the following manner: a 1.2 cm x 1.6 cm stencil made of polystyrene is laid gently on the back and a saline-wetted sterile gauze could be used to partially wet the skin (allowing even application of the immunizing solution), the immunizing solution could then be applied with a pipet to the area circumscribed by the stencil to yield a 2 cm^2 patch of immunizing solution. Care could be used not to scrape or rub the skin with the pipet tip. The immunizing solution could be spread around the area to be covered with the smooth side of the pipet tip. Alternatively, the immunizing solution could be place directly on the skin without wetting or with wetting without the use of a stencil.

The immunizing solution (between about 5 µl and about 200 µl) could be left on the back of the mouse for 60-120 minutes. At the end of the immunization period, the mouse could be held gently by the nape of the neck and the tail under a copious stream of lukewarm tap water, and washed for 10 seconds. The mouse could then be gently patted dry with a piece of sterile gauze and a second washing could then be performed for 10 seconds; the mouse could then be patted dry a second time and left in the cage. The mice would appear to exhibit no adverse effects from the anesthesia, immunization, washing procedure, or toxicity from the exotoxins. No skin irritation, swelling or redness would be seen after the immunization and the mice would appear

to thrive. Immunization using the ear could be performed as described above except that fur would not be removed prior to immunization.

Antigen

The following antigens could be used for immunization and ELISA, and could be mixed using sterile PBS or normal saline. Cholera toxin or CT (List Biologicals, Cat #101B, lot #10149CB), CT B subunit (List Biologicals, Cat #BT01, lot #CVXG-14E), CT A subunit (List Biologicals, Cat #102A, lot #CVXA-17B), CT A subunit (Calbiochem, Cat #608562); pertussis toxin, salt-free (List Biologicals, lot #181120a); tetanus toxoid (List Biologicals, lots #1913a and #1915a); Pseudomonas exotoxin A (List Biologicals, lot #ETA25a); diphtheria toxoid (List Biologicals, lot #15151); heat-labile enterotoxin from E. coli (Sigma, lot #9640625); bovine serum albumin or BSA (Sigma, Cat #3A-4503, lot #31F-0116); and Hemophilus influenza B conjugate (Connaught, lot#6J81401).

ELISA - IgG(H+L)

Antibodies specific for CT, LT, ETA, pertussis toxin, diphtheria toxoid, tetanus toxoid, Hemophilus influenza B conjugate, and BSA could be determined using ELISA in a technique similar to Glenn et al. (1995).

Example 22

Activation of LT may be performed by incubating the LT with trypsin (or trypsin immobilized on beads) with or without reducing agents (e.g., dithiotheritol) to break the disulphide bonds near the trypsin cleavage site, under standard reaction conditions. Native LT can be activated by incubation of 100 µg of protein with 0.1 µg trypsin in a total reaction volume of 100 µl for 45 min at 37°C. Alternatively, the trypsin can be fixed to beads and the LT may be eluted over the trypsin beads. Trypsin cleavage can be demonstrated by SDS-PAGE (Laemilli, U.K., 1970, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227:680-685). LT either treated or not treated with trypsin can be mixed with buffer containing dithiothreitol, and heated to 100°C for 5 min prior to SDS-PAGE analysis. Trypsin-treated LT could have a proteolytic fragment of 21K daltons consistent with trypsin cleavage of the A1 and A2, allowing the A1 subunit to ADP-ribosylate G proteins and therefore exert its toxic effects whereas untreated LT would demonstrate a band at 28K daltons, consistent with an intact A subunit. Activation can further be demonstrated in the mouse Y-1 cell assay in which native LT would be 1,000 fold less active than CT, but trypsin-treated LT would be equally as active as CT. Activation can also be demonstrated using an enzymatic assay, the NAD:agmatine ADP-ribosyltransferase assay. In such an assay, non-trypsin-treated LT would be expected to show low or undetectable activity whereas trypsin-treated LT would be expected to show similar activity as that demonstrated by CT.

Example 23.

Transcutaneous immunization may be more useful if the immunization can be performed over a short period of time. It may be useful for example for an immunization to be performed during a routine clinic visit lasting 30 minutes. In this example we show that transcutaneous immunization can be performed in hydrated, alcohol swabbed skin in such a short period.

C57BL/6 mice 6 to 8 weeks of age were anesthetized and shaved as described in the "immunization procedure". On the day of immunization the backs of the mice were wiped with isopropanol. After the alcohol had evaporated (approximately 10 minutes), 200 µl of water was applied to the back for hydration. 15 minutes later the immunization solution was applied to the back and left for the specified period of time. Removal of excess antigen was conducted as described in the "immunization procedure." Mice were immunized with CT alone (100 µg in 50 µl) at d0 and with CT plus DT (100 µg each in 100 µl volume) at 4, 6 and 9 weeks. Twelve weeks after the primary immunization the animals were bled and the anti-DT titers determined using an ELISA as described above for "ELISA IgG (H+L)". The results are shown in Table 23.

Anti-DT titers were clearly elevated in serum from all of the animals immunized with CT and DT when compared with titers in serum from the same animals prior to immunization (prebleed). Maximal effects of immunization appeared to occur in animals vaccinated for a period of 60 minutes although the titers were similar at 30 and 120 minutes. Fifteen minutes of immunization seemed less efficient as the titers in this group were approximately 10 fold less than that observed in the 30, 60 and 120 minute groups. Thus it appears that TCI can be achieved within 15 minutes of antigen application.

Table 23. Effect of the duration of antigen application on humoral immunity induced by transcutaneous immunization.

ear tag #	duration of immunization	anti-DT IgG (ELISA units)		
		prebleed	12 weeks	geomean
361	15 min	6	214	300
362	15 min		664	
363	15 min		314	
364	15 min		181	
365	15 min		1594	
366	30 min	8	11953	13445
367	30 min		32478	
368	30 min		24346	
369	30 min		3457	
370	30 min		99776	
371	60 min	12	75787	107963
372	60 min		200768	
373	60 min		102592	
374	60 min		87034	
375	60 min		9210	
376	120 min	4	48132	48202
377	120 min		99362	
378	120 min		37308	
379	120 min		30255	
380	120 min		25149	

All publications referred to in this application are incorporated by reference herein as indicative of the state of the art.

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What we claim is:

1. A method for inducing an enhanced therapeutically effective immune response in an subject comprising:

a. pretreating an area of the skin of said subject; and

b. applying to said pretreated area a formulation comprising:

1) a therapeutically effective amount of at least one antigen,

2) at least one adjuvant present in an amount effective to promote an immune response to said at least one antigen, and

3) a pharmaceutically acceptable carrier to the skin of said subject, wherein said pretreated area is not perforated.

2. The method of claim 1, wherein said pretreating enhances skin penetration by said formulation.

3. The method of claim 1, wherein said carrier is a patch.

4. The method of claim 3, wherein said patch is selected from the group consisting of an occlusive dressing, an nonocclusive dressing, a hydrogel dressing and a reservoir dressing.

5. The method of claim 1, wherein said pretreating comprises swabbing said pretreated area with a swab.

6. The method of claim 5, wherein said swab is comprised of material selected from the group consisting of cotton, nylon, wool and combinations thereof.

7. The method of claim 5, wherein said swab is treated with an alcohol or a composition containing alcohol.

8. The method of claim 5, wherein said swab is treated with acetone or a composition containing acetone.

9. The method of claim 1, wherein said pretreating comprises applying a detergent or a detergent solution to said pretreated area.

10. The method of claim 5, wherein said swab is treated with a detergent or a detergent solution.

11. The method of claim 5, wherein said pretreating comprises applying a depilatory formulation, leaving said formulation on said pretreated area for a period of about 0.1 – 30 minutes.

12. The method of claim 11, wherein said period is preferably about 4 - 20 minutes.

13. The method of claim 11, wherein said period is more preferably about 12 minutes.

14. The method of claim 1, wherein said pretreating comprises applying a keratinolytic formulation, leaving said formulation on said pretreated area for a period of about 0.1 – 30 minutes.

15. The method of claim 14, wherein said keratinolytic formulation is salicylate.

16. The method of claim 14, wherein said period is about 4 - 20 minutes.

17. The method of claim 14, wherein said period is about 10 minutes.

18. The method of claim 1, wherein said pretreating comprises disrupting the surface layer of said pretreated area with a disrupting device.

19. The method of claim 18, wherein said disrupting device is selected from the group consisting of an emory board, an abrasive pad, a TB tine testing device, a gas powered gun, a microneedle device and adhesive tape.

20. The method of claim 1, wherein said adjuvant is at least one of the members selected from the group consisting of bacterial DNA, cytokines, chemokines, tumor necrosis factor alpha, genetically altered toxins, chemically conjugated toxins and lipopolysaccharides.

21. The method of claim 20, wherein said therapeutically effective immune response results in LN cell proliferation.

22. The method of claim 20 wherein said adjuvant is a combination of at least two of the adjuvants selected from the group consisting of bacterial DNA, CpG, cytokines, chemokines, tumor necrosis factor alpha, genetically altered toxins, chemically conjugated toxins and lipopolysaccharides.

23. A method for inducing an enhanced therapeutically effective immune response in an subject comprising:

a. applying to said pretreated area a formulation comprising:

1) a therapeutically effective amount of at least one antigen,

2) at least one adjuvant present in an amount effective to promote an immune response to the antigen, and

3) a pharmaceutically acceptable carrier to the skin of said subject, wherein said pretreated area is not perforation; and

b. administering a separate antigen formulation to said subject.

24. The method of claim 23, wherein said separate antigen formulation is administered at a time after said applying of said formulation to said pretreated area, wherein said separate antigen formulation provides a further immune response in said subject.

25. The method of claim 23, wherein said separate antigen formulation is administered at a time before said applying of said formulation to said pretreated area, wherein said separate antigen formulation provides a further immune response in said subject.

26. The method of claim 1, wherein said adjuvant is applied to the skin at one site on said subject and said antigen is applied to a second site on the skin on said subject and at least one of said first site and said second site is on said pretreated area.

27. The method of claim 26, wherein said application of said antigen and said adjuvant occur about simultaneously.

28. A method for inducing an enhanced therapeutically effective immune response in an subject comprising:

a. pretreating an area of the skin of said subject;

b. administering an effective amount of at least one antigen; and administering an effective amount of at least one adjuvant, wherein at least one of said antigen and said adjuvant are administered on said pretreated area and said pretreated area is not perforated.

29. The method of claim 28, wherein said antigen is administered to said pretreated area and said adjuvant is administered by a process selected from the group consisting of intramuscular injection, oral, nasal and rectal.

30. The method of claim 28, wherein said adjuvant is administered to said pretreated area and said antigen is administered by a process selected from the group consisting of intramuscular injection, oral, nasal and rectal.

31. The method of claim 1, wherein said antigen presents on a cell surface of a Langerhans cell to a lymphocyte, thereby inducing the immune response in the organism.

32. The method of claim 1, wherein exposure to said adjuvant causes migration of the Langerhans cell to a lymph node.

33. The method of claim 1, wherein exposure to said adjuvant signals the Langerhans cell to mature into a dendritic cell.

34. The method of claim 1, wherein the antigen is derived from a source selected from the group consisting of a pathogen, a tumor cell and a normal cell.

35. The method of claim 1, wherein the antigen is derived from a pathogen selected from the group consisting of a bacteria, virus, fungus and parasite.

36. The method of claim 1, wherein the antigen is a selected from the group consisting of a tumor antigen, an autoantigen, an allergen and a biological warfare agent.

37. The method of claim 1, wherein the antigen is selected from the group consisting of carbohydrate, glycolipid, glycoprotein, lipid, lipoprotein, phospholipid, and polypeptide.

38. The method of claim 1, wherein the formulation further comprises an attenuated live virus and the antigen is expressed by the attenuated live virus.

39. The method of claim 1, wherein the antigen is multivalent.

40. The method of claim 31, further comprising activating the Langerhans cell to increase major histocompatibility complex class II expression.

41. The method of claim 31, wherein the adjuvant activates the Langerhans cell.

42. The method of claim 1, wherein the adjuvant enhances antigen presentation to a lymphocyte.

43. The method of claim 1, wherein the adjuvant is an ADP-ribosylating exotoxin.

44. The method of claim 43, wherein the adjuvant is cholera toxin (CT) or cholera toxin B subunit (CTB).

45. The method of claim 43, wherein the adjuvant is *E. coli* heat-labile enterotoxin (LT) or pertussis toxin.

46. The method of claim 43, wherein the adjuvant in said formulation is provided as a nucleic acid encoding an ADP-ribosylating exotoxin.

47. The method of claim 1, wherein the antigen in the formulation is provided as a nucleic acid including a sequence encoding the antigen.

48. The method of claim 47, wherein the nucleic acid is non-integrating and non-infectious.

49. The method of claim 47, wherein the nucleic acid further includes a regulatory region operably linked to the sequence encoding the antigen.

50. The method of claim 1, wherein the formulation is a gel or emulsion or ointment.

51. The method of claim 1, wherein the formulation is applied to intact skin covering more than one draining lymph node field.

52. An article for vaccine administration comprising a patch suitable for adhesion to the skin and a vaccine formulation including at least one adjuvant, at least one antigen and a skin penetration enhancer.

53. The article of claim 52, wherein said skin penetration enhancer is selected from the group consisting of an alcohol, acetone, a detergent, a depilatory and a keratinolytic.

54. The article of claim 52, wherein said alcohol or said acetone are combined with a swab.

55. A method of treating a disease of a subject, the method comprising:

a. pretreating an area of the skin of said subject, wherein said pretreatment enhances efficacy of said treatment; and

b. applying to said pretreated area a formulation comprising:

1) a therapeutically effective amount of at least one antigen,

2) at least one adjuvant present in an amount effective to promote an immune response to said at least one antigen, and

3) a pharmaceutically acceptable carrier to the skin of said subject, wherein said pretreated area is not perforated.

56. A method of preventing a disease of a subject, the method comprising:

a. pretreating an area of the skin of said subject, wherein said pretreatment enhances efficacy of said preventing; and

b. applying to said pretreated area a formulation comprising:

1) a therapeutically effective amount of at least one antigen,

2) at least one adjuvant present in an amount effective to promote an immune response to said at least one antigen, and

3) a pharmaceutically acceptable carrier to the skin of said subject, wherein said pretreated area is not perforated.

57. A method of protecting a subject from exposure to an antigen, the method comprising:

a. pretreating an area of the skin of said subject, wherein said pretreatment enhances efficacy of said protection; and

b. applying to said pretreated area a formulation comprising:

1) a therapeutically effective amount of at least one antigen,

2) at least one adjuvant present in an amount effective to promote an immune response to said at least one antigen, and

3) a pharmaceutically acceptable carrier to the skin of said subject, wherein said pretreated area is not perforated.

58. A composition comprising:

at least one antigen;

at least one adjuvant; and,

at least one skin penetration enhancer, wherein said composition when applied to intact skin induces a immune response specific to said antigen.

59. The composition of claim 58, wherein said skin penetration enhancer is selected from the group consisting of an alcohol, acetone, a detergent, a depilatory and a keratinolytic.

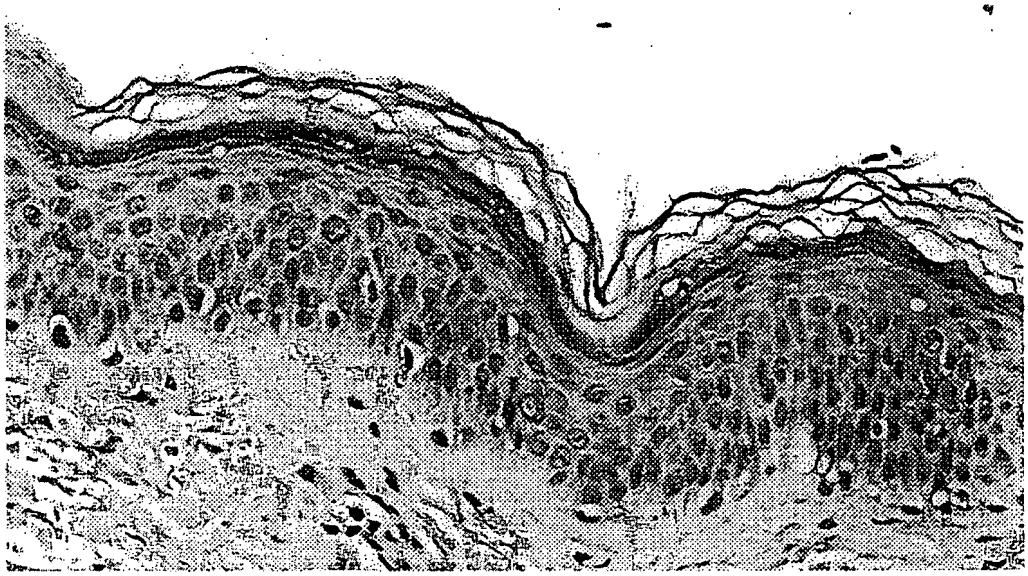

FIG. 1A

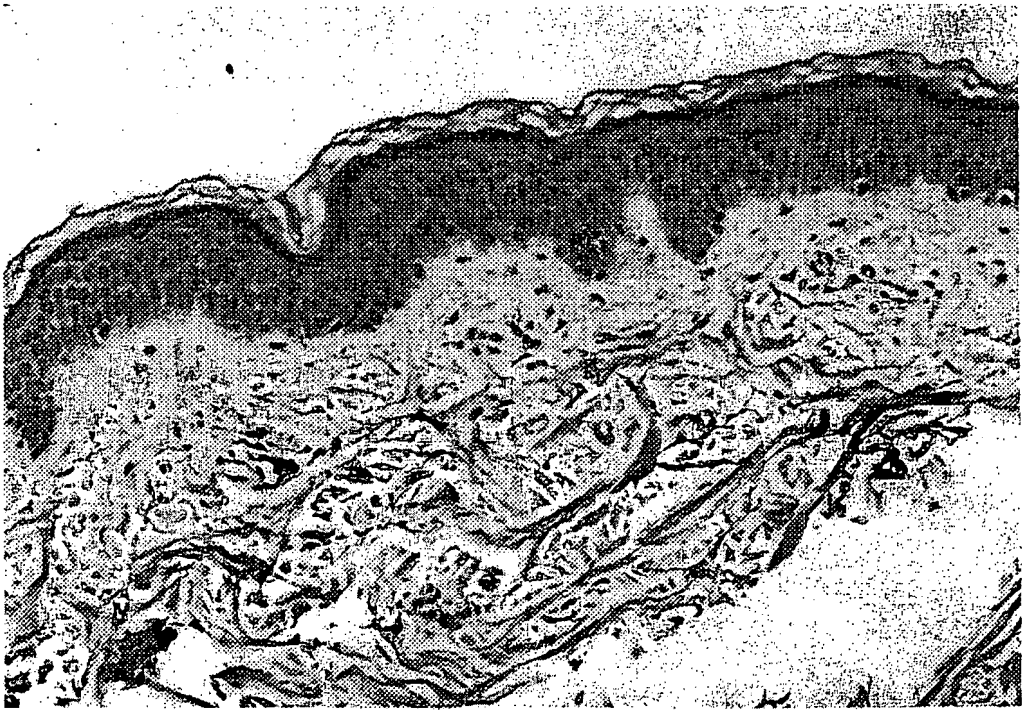

FIG. 1B

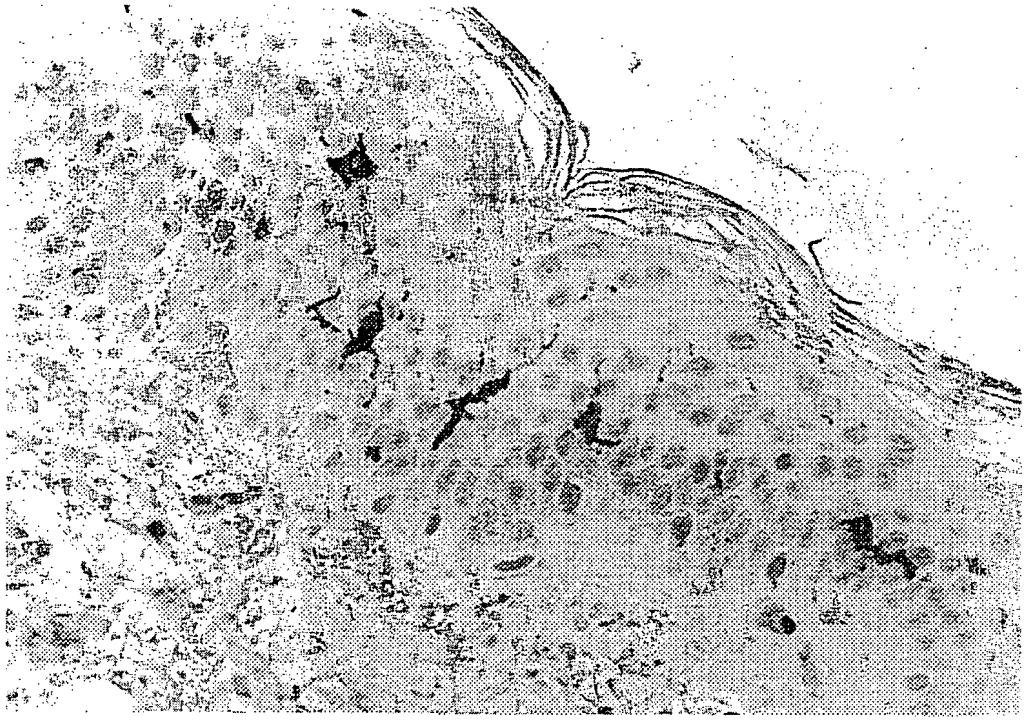

FIG. 1C

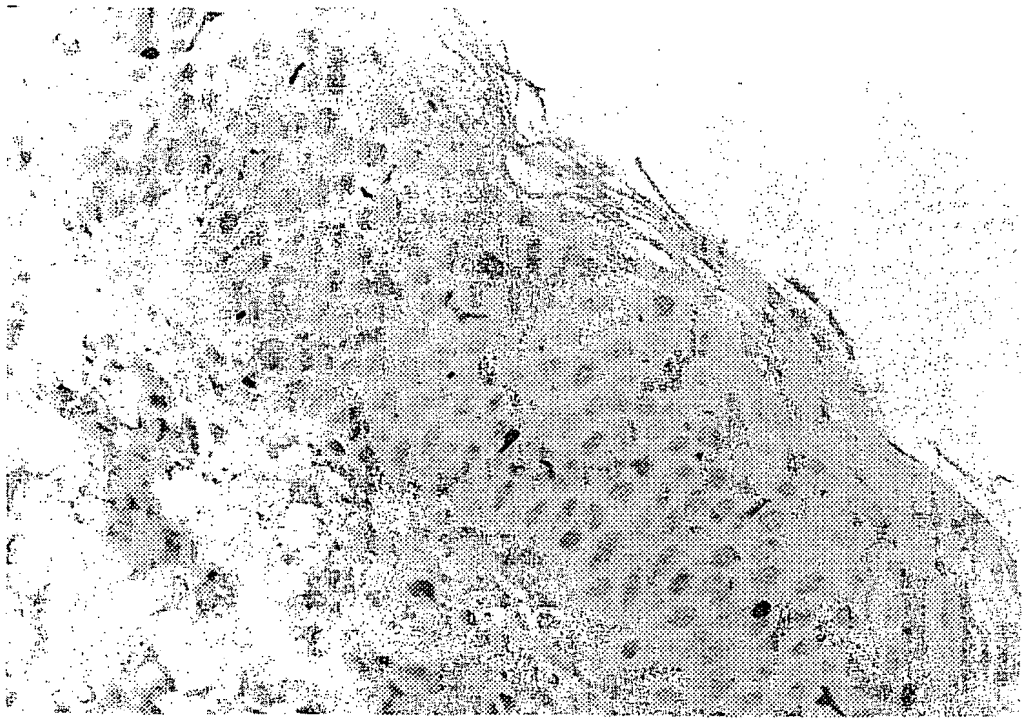

FIG. 1D

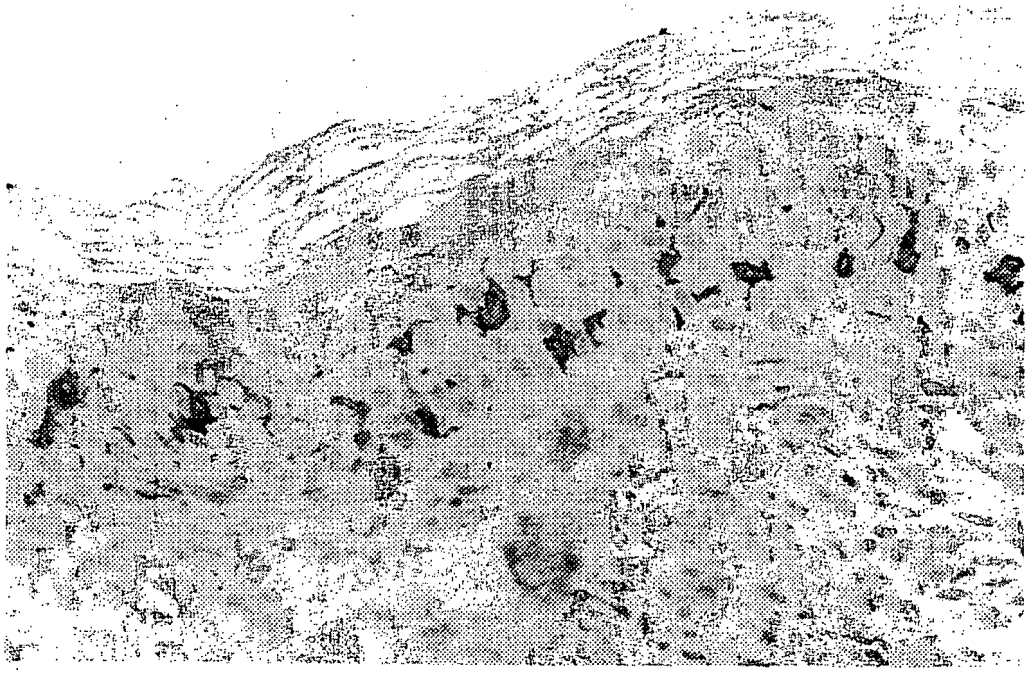

FIG. 1E

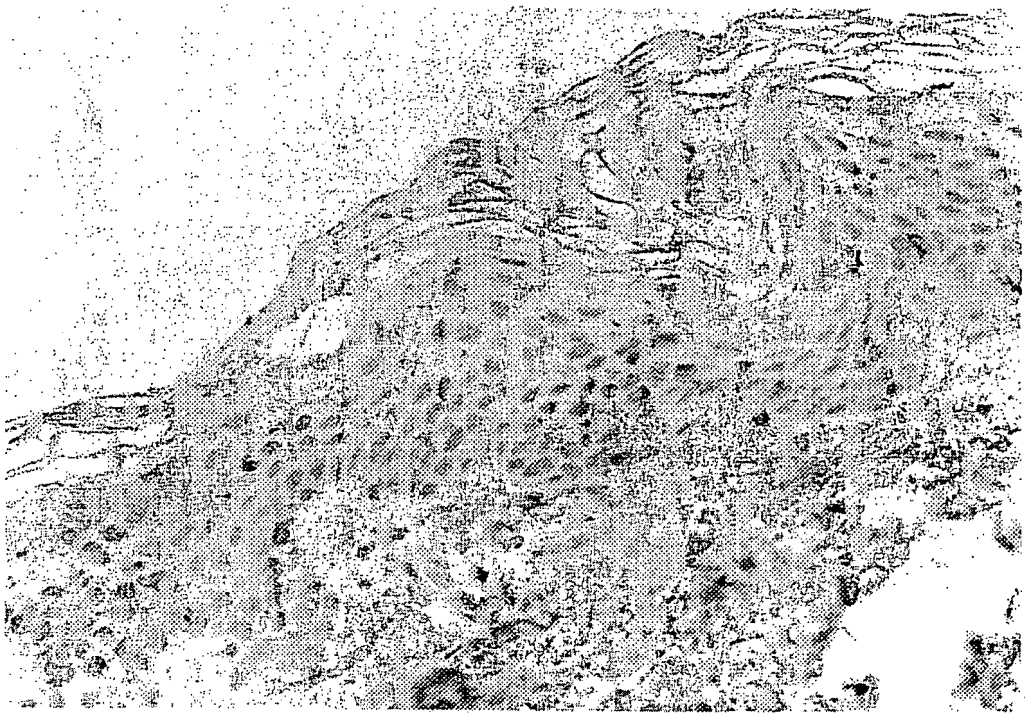

FIG. 1F

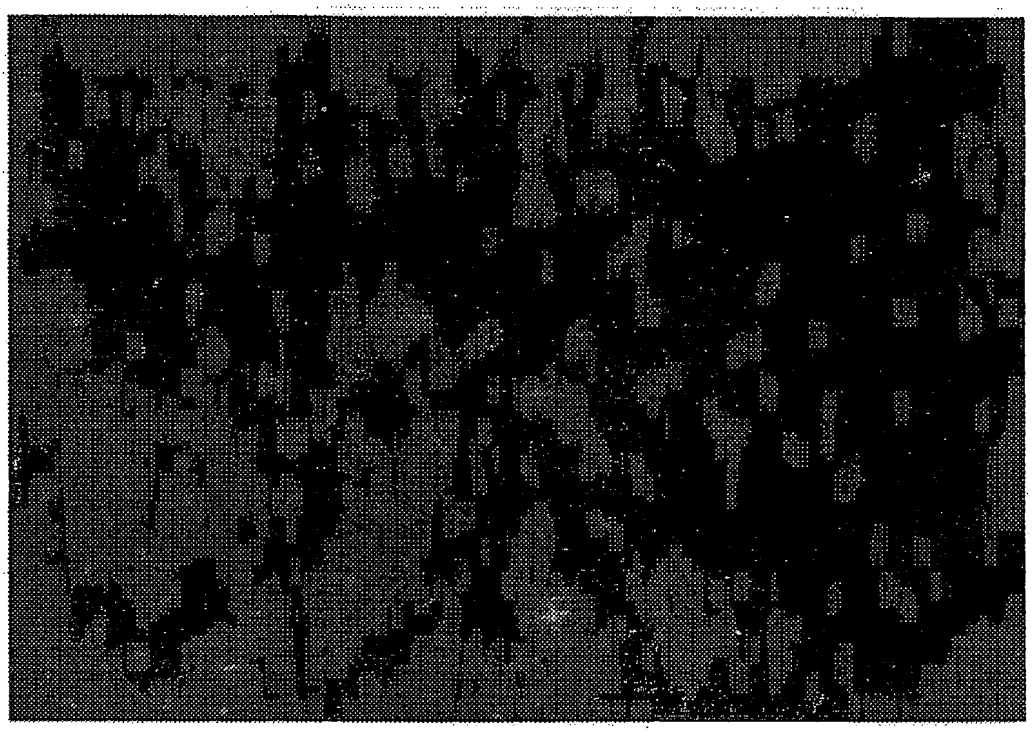

FIG. 2A

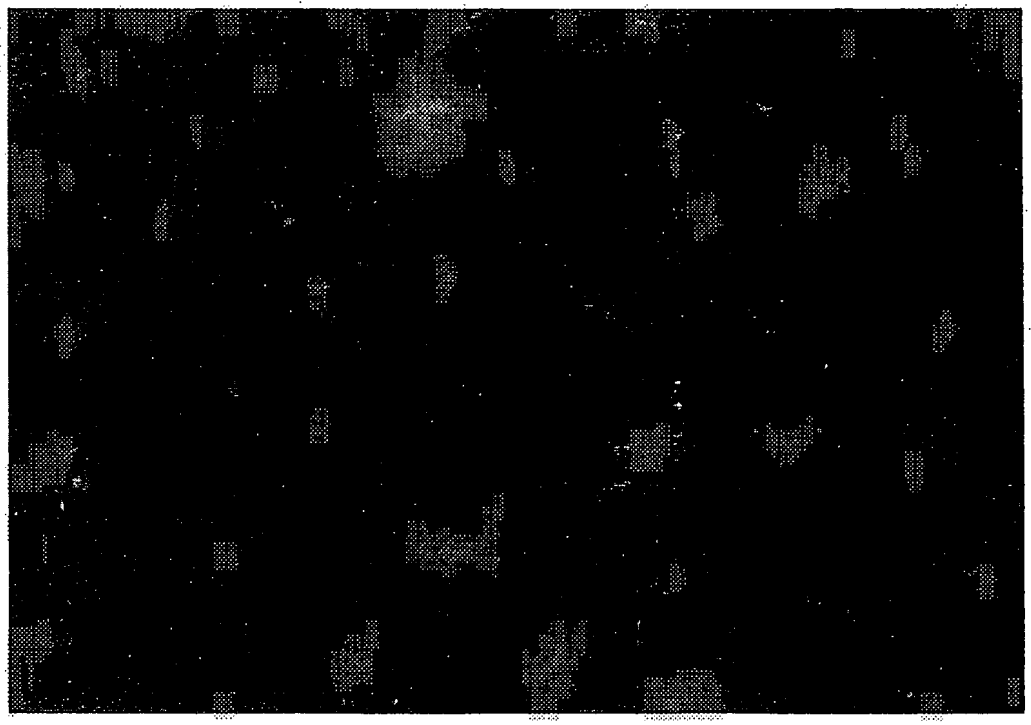

FIG. 2B

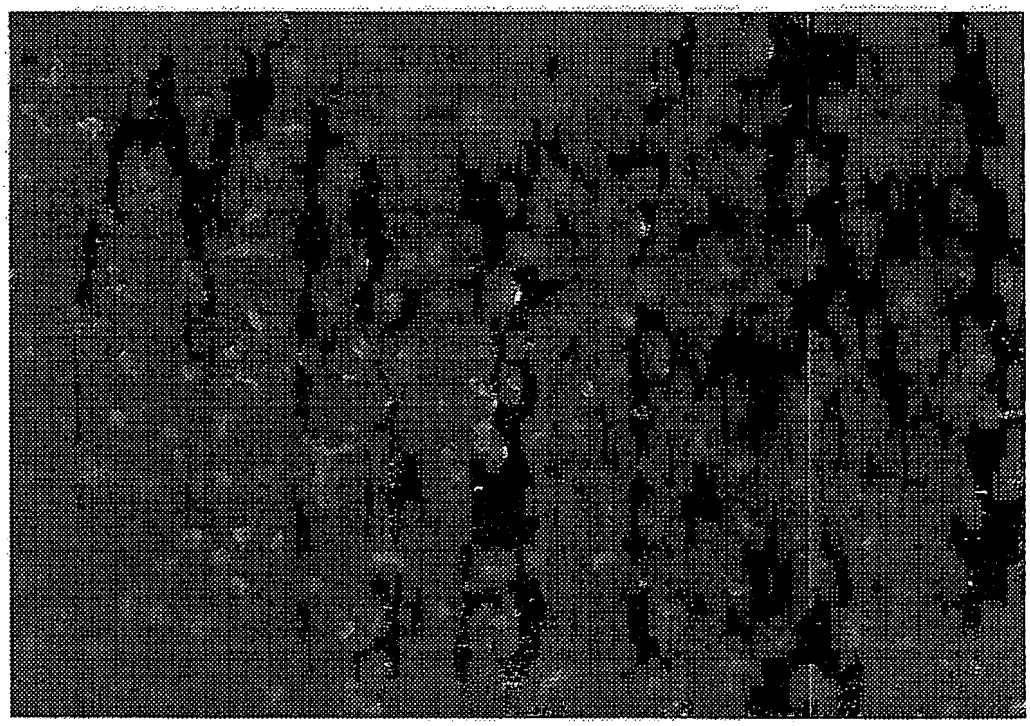

FIG. 2C

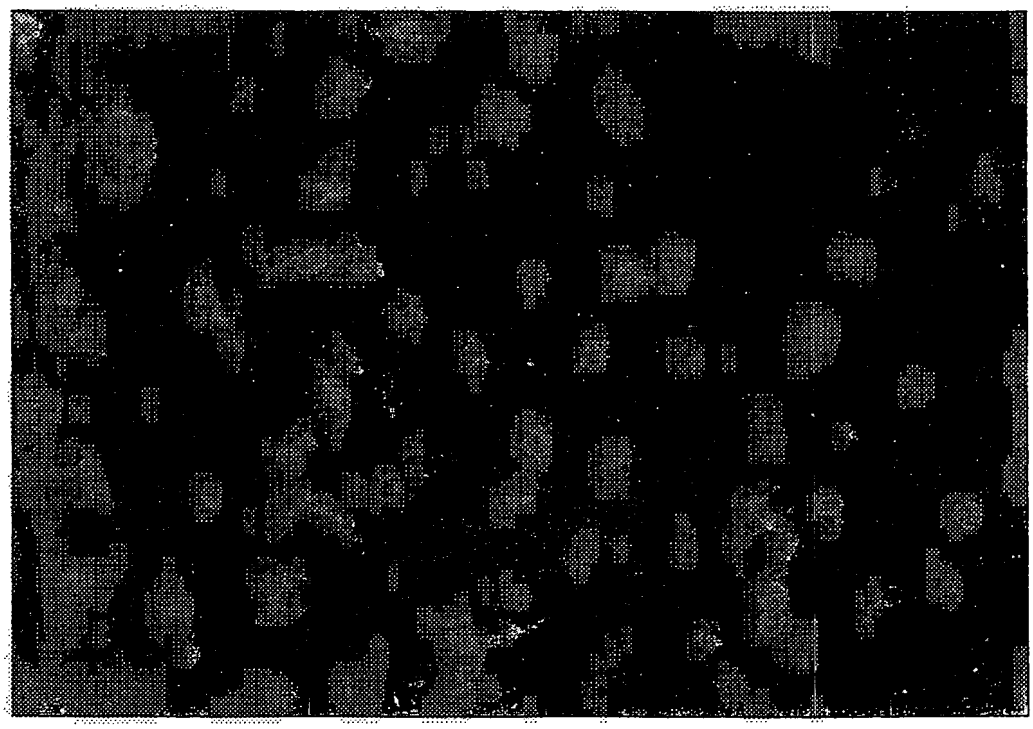

FIG. 2D

INTERNATIONAL SEARCH REPORT

Interi nal Application No
PCT/US 99/04128

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K39/39 A61K9/00 C12N15/11 //(A61K39/39,A61K38:16), (A61K39/39,A61K39:02),(A61K39/39,A61K39:106)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	TANG, DE-CHU ET AL: "Vaccination onto bare skin" NATURE (LONDON) (1997), 388(6644), 729-730 , XP002110051 cited in the application the whole document ---	1-59
A	PAUL, AMLA ET AL: "Transdermal immunization with large proteins by means of ultradeformable drug carriers" EUR. J. IMMUNOL. (1995), 25(12), 3521-4 , XP002110052 cited in the application the whole document ---	1-59

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 July 1999	13.08.99

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Mennessier, T

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inten nal Application No
PCT/US 99/04128

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,Y	GLENN G M ET AL: "Skin immunization made possible by cholera toxin [letter]." NATURE, (1998 FEB 26) 391 (6670) 851. , XP002110053 the whole document	1-59
P,Y	WO 98 20734 A (ALVING CARL R ;US HEALTH (US); GLENN GREGORY M (US)) 22 May 1998 (1998-05-22) cited in the application the whole document	1-59
T	GLENN G M ET AL: "Transcutaneous immunization with bacterial ADP-ribosylating exotoxins as antigens and adjuvants." INFECTION AND IMMUNITY, (1999 MAR) 67 (3) 1100-6. , XP002110054 cited in the application the whole document	1-59
T	VASSELL, R. (1) ET AL: "Activation of Langerhans cells following transcutaneous immunization." FASEB JOURNAL, (MARCH 12, 1999) VOL. 13, NO. 4 PART 1, PP. A633. MEETING INFO.: ANNUAL MEETING OF THE PROFESSIONAL RESEARCH SCIENTISTS FOR EXPERIMENTAL BIOLOGY 99 WASHINGTON, D.C., USA APRIL 17-21, 1999 , XP002110055 the whole document	1-59
T	GLENN, GREGORY M. ET AL: "Advances in vaccine delivery: transcutaneous immunisation" EXPERT OPIN. INVEST. DRUGS (JUNE 1999), 8(6), 797-805 , XP002110056 the whole document	1-59

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 99/04128

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 1-51 and 55-57 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the formulation.

2. [X] Claims Nos.: 1-59 (partly)
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 99/04128

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1-59 (partly)

The independent claims are written in an extremely large manner with a number of unclarities. Particularly, the terms "pretreating/pretreated" and "adjuvant" as well as the phrase "skin penetration enhancer" as referred to in said claims are used without any restriction as to their meaning with the result that a complete meaningful search into the state on the art on the basis of all the claims as originally filed is not possible. Nevertheless, a search has been carried out on the basis of the whole set of claims with the following limitations relying on the disclosure of the invention in the description and the aspects of the invention for which an actual support exists therein:

- in claims 1, 23, 28, 52, 55, 56, 57 and 58, it has been considered that the adjuvant is limitedly an ADP-ribosylating exotoxin. In dependent claim 20, the same limitation has been made.

- in claim 20 (dependent on claim 1) only that embodiment has been taken into consideration according to which the adjuvant of claim 1 is a genetically detoxified mutant of an ADP-ribosylating-exotoxin (see example 8).

- in dependent claim 22 only combinations of an ADP-ribosylating exotoxin with IL-2 (see example 9), TNF-alpha (see example 9), and the nucleic sequence of formula TCCATGACGTTCCTGACGTT (see example 7) have been taken into consideration.

- in claim 1, 23, 28, 55, 56 and 57, it has been considered that the pretreatment referred to therein is limitedly carried out with a view to enhancing skin penetration by the formulation by removing or modifying a portion of the stratum corneum or the superficial epidermis (as can be derived from the description and ilustrated by Example 5).

- in claims 52 and 58, it has been considered that the skin penetration enhancer has to be chosen in such a way that it is capable of only removing or modifying a portion of the stratum corneum or the superficial epidermis.

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter. ɔnal Application No
PCT/US 99/04128

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9820734 A	22-05-1998	US 5910306 A	08-06-1999
		AU 5265298 A	03-06-1998